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HORSERADISH PEROXIDASE CATALYZED OXIDATION OF AROMATIC AMINES AND MYELOPEROXIDASE REACTIONS WITH HYDROGEN PEROXIDE

by

JIN TONY HUANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled "Horseradish Peroxidase Catalyzed Oxidation of Aromatic Amines and Myeloperoxidase Reactions with Hydrogen Peroxide" submitted by Jin Tony Huang in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Chemistry.

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This thesis is dedicated to my wife, Helen,

my daughter, Laura, and my son, Jeff.

ABSTRACT

PART I

The horseradish peroxidase (HRP) catalyzed oxidation of aromatic amines was investigated in order to obtain information of potential relevance to the *in vivo* activation of carcinogenic arylamines. Firstly, the kinetics of oxidation of a series of monosubstituted anilines by HRP compound II was studied. It was found that the oxidation of these anilines can be correlated by the Hammett $\sigma\rho$ equation with the value of ρ, the susceptibility factor, being -6.0 ± 0.7. This result, combined with the result of a pH dependence study of these reactions, suggests that the first step of the oxidation is the removal of a hydrogen atom from the amino group. However, the rates of oxidation of the model compound for carcinogenic arylamines, 2-aminofluorene (2-AF) by HRP compounds I and II, $(1.1 \pm 0.5) \times 10^9$ M^{-1} s^{-1} and $(1.7 \pm 0.2) \times 10^8$ M^{-1} s^{-1}, are about two orders of magnitudes higher than those expected on the basis of the aforementioned Hammett $\sigma\rho$ relationship, implying a different mechanism. It is therefore proposed that a cation radical is formed from 2-AF in the first step of the reaction which is stabilized by the extensive π-conjugation system of 2-AF. These results are of significance in that they provide a potential explanation for the high degree of carcinogenesis found in the polycyclic aromatic amines compared to monocyclic aromatic amines.

PART II

A new mechanism for the reactions of myeloperoxidase (MPO) with hydrogen peroxide is proposed based on both qualitative and quantitative studies of these reactions. According to this mechanism, MPO compound I is formed from native MPO and hydrogen peroxide in a reversible reaction. MPO compound I further reacts with hydrogen peroxide to form compound II and superoxide. Rate constants obtained from stopped-flow experiments are $(2.8 \pm 0.5) \times 10^7\ M^{-1}\ s^{-1}$ for the formation of compound I and $(20 \pm 4)\ s^{-1}$ for the reverse reaction, namely the decomposition of compound I to native MPO and hydrogen peroxide, and $(1.6 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ for the reaction of compound I with hydrogen peroxide. Superoxide, once formed from the reduction of compound I by hydrogen peroxide, also reduces compound I to compound II through an intermediate complex. Relevant constants were obtained through a combination of calculations based on kinetic measurements and optimizing simulations. They are $2.5 \times 10^9\ M^{-1}\ s^{-1}$ and $100\ s^{-1}$ for the formation and the dissociation of the compound I - superoxide complex, and $0.1\ s^{-1}$ for the conversion of the intermediate complex to compound II and molecular oxygen. The overall kinetics predicted in terms of the postulated mechanism and the rate constants obtained by computer simulation is consistent with the experimentally observed behavior of MPO in the presence of hydrogen peroxide.

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PART I

HORSERADISH PEROXIDASE CATALYZED OXIDATION OF AROMATIC AMINES

CHAPTER ONE

INTRODUCTION

1.1 HORSERADISH PEROXIDASE

1.1.1 Peroxidases: an overview

Peroxidases, by definition, are the enzymes which catalyze the oxidation of molecules by peroxides. As recommended by the Nomenclature Committee of the International Union of Biochemistry, peroxidases are classified as a sub-group of oxidoreductases with EC number of 1.11.1.

"It is probably true to say that among all the enzymes few have attracted more attention than has peroxidase. This widely distributed enzyme has claimed the attention of physical chemists, organic chemists, biochemists and physiologists for many years. ··· It may be said with justification that our present knowledge is very imperfect in many substantial ways and in this state of uncertainty it may well be argued that this is not yet the time for a review on peroxidase."

The above paragraph is excepted from the book, "PEROXIDASE", which was written by Saunders, Holmes-Siedle and Stark nearly thirty years ago (1). Today, when I am writing the first section of this thesis, I still have the same feeling as expressed in these statements.

Peroxidases can be found almost everywhere in the biological world, from cells in microorganisms and plants to animal tissues. Their wide distribution in nature on one hand makes it difficult to fully understand their biological functions but on the other hand provides evidence for their universal biological importance. Horseradish peroxidase, turnip peroxidase and Japanese radish peroxidase are examples of plant peroxidases (2). It has been proposed that peroxidases are key molecules in the rapid adaption process to changes in their environment for the whole plants or for some of their organs (3). Peroxidases found in animal tissues and fluids have diversified physiological functions. Myeloperoxidase of neutrophils plays a key role in the phagocytic process (4). Lactoperoxidase contributes to antibacterial activities of a number of mammalian exocrine gland secretions (5). Thyroid peroxidase is involved in the biosynthesis of the hormones thyroxine and triiodothyronine (6-8). The initial two reactions in the biosynthesis of prostaglandins are catalyzed by the heme protein, prostaglandin H synthase (9). After the cyclooxygenase component of prostaglandin H synthase oxygenates arachiodonic acid to PGG_2, a

hydroperoxy endoperoxide, its peroxidase component reduces PGG_2 to an alcohol, PGH_2. Apart from the plant and animal sources, many fungi, algae, bacteria, and microorganisms also contain peroxidases. Chloroperoxidase is isolated from the fungus, *Caldariomyces fumago* (10-12). Bromoperoxidase is isolated from marine algae (13). Chloroperoxidase and bromoperoxidase have unique reactivities to oxidize effectively inorganic chloride and bromide ions (11-13). Cytochrome *c* peroxidase from aerobically grown yeast has a high specific activity toward ferrocytochrome *c* (14,15). Two peroxidases isolated from the white rot fungus *Phanerochaete chrysosporium* , manganese peroxidase and lignin peroxidase, were characterized recently (16-19). Investigations have shown that the two peroxidases are actively involved in lignin biodegradation (16,20,21). A plausible hypothesis for the function of all peroxidases is that they were first used by primordial organisms in their defence mechanism against oxidative damage by toxic oxygen species and that once survival from oxidation was firmly established, more specialized functions evolved (4).

Generally, peroxidases consist of a heme group and glycoprotein. The heme prosthetic group contributes to the catalytic function of the enzyme in two ways: (i) the iron activates the heterolytic cleavage of peroxides and stores one oxidizing equivalent obtained from the oxidizing substrate, either a hydroperoxide or a peroxy acid, and (ii) the porphyrin regulates the oxidation-

reduction potential and stores the other oxidizing equivalent. While all of the peroxidases isolated so far from plants and microorganisms contain ferriprotoporphyrin IX (Fig. 1.1) as the prosthetic group, peroxidases from animal sources present some exceptions. Usually they have prosthetic groups different from ferric protoporphyrin IX (2). The heme of myeloperoxidase has been suggested to be a modified protoporphyrin in which the methyl group at position 8 or 5 is replaced by a formyl group (22). The heme structure of lactoperoxidase has been controversial. A recent study has suggested a heme almost identical to protoporphyrin IX except for the substitution of a -CH_2SH group for the -CH_3 group at the C-18 position (23). For both myeloperoxidase and lactoperoxidase, it appears that a disulfide bond is formed between heme and a protein cysteinyl residue (24). The structure of the prosthetic group and its interaction with the protein moiety result in a unique set of catalytic properties for each peroxidase.

In spite of the large number of peroxidases discovered in nature, similarities exist among peroxidases from different sources. Horseradish peroxidase is the most intensively studied enzyme hence it is often used as a model of other peroxidases.

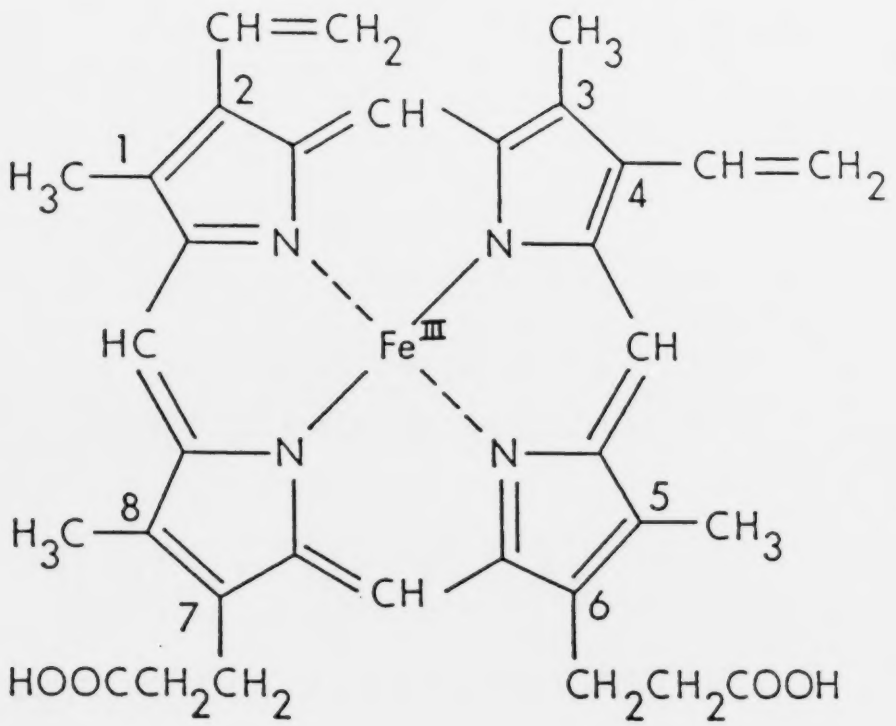


Figure 1.1. Structure of ferriprotoporphyrin IX: the prosthetic group for most peroxidases.

1.1.2 Structure and properties of native HRP

There are at least seven major isoenzymes reported for HRP. Only the isoenzyme of isoelectric point ~ 9 (isoenzyme C or IIb) will be discussed here because it is the dominant isoenzyme of most commercial preparations and the isoenzyme used in this work.

Native HRP contains a ferriprotoporphyrin IX as its prosthetic group (Fig. 1.1). The ligands at coordination positions 1 through 4 for the ferric ion are the four nitrogen atoms of the pyrrole rings. The fifth coordination position is occupied by an imidazole group of a histidine residue, which is referred to as the proximal histidine (25). The proposed presence of water at the sixth position, which is located at the distal side of the heme plane, remained controversial for many years. The controversy appears to have been resolved. Results of ESR and photolysis studies indicate the absence of water (26-28), so do the comparisons of optical spectra of HRP with metmyoglobin whose crystal structure is known (29). However, a weakly bonded water molecule cannot be excluded.

Complete amino acid sequences of HRP have been determined (30). Besides the heme prosthetic group, HRP has two Ca^{2+} and 308 amino acid residues including four disulfide bridges, in a polypeptide chain that carries 8 neutral carbonhydrate side chains for a total molecular weight close to 44,000.

Calcium particularly contributes to maintaining the structural conformation of the protein. The primary amino acid sequence of HRP confirmed the presence of the proximal histidine and demonstrated that the active site occurred in a hydrophobic region of the amino acid sequence (30,31). The histidine and arginine residues were found on the distal side. Amino acid sequences of other peroxidases have been studied, which indicate that the distal and proximal histidine and the distal arginine are conserved among almost all heme peroxidases (32,33).

The number of unpaired electrons of a transition metal ion, hence the spin state is dependent upon its oxidation state, coordination number and the field strength of its ligands (29,34). The ferric ion in native HRP is penta-coordinated. There are two opposing effects on the ligand field strength. While the absence of a sixth ligand would decrease the d-orbital splitting, the fairly strong coordination of the fifth ligand could pull the iron out of the porphyrin plane, yielding a distorted square pyramidal complex. It has been established that the ferric ion of native HRP has a predominantly high spin (S=5/2), probably mixed with a small part of intermediate spin (S=3/2) (35,36).

Due to its unique structure and properties stated above, HRP can be distinguished spectroscopically from cytochrome P-450 (oxygenase) in which the heme iron has a cysteinate as the fifth ligand (37), from cytochrome b_5 (electron carrier) in which the heme iron is a low spin ferric ion (38), from oxy-

and deoxymyoglobin (oxygen binder) in which the heme iron is in the ferrous state (39), and from metmyoglobin in which the heme iron is hexa-coordinated with water in the sixth coordination position (28).

1.1.3 Enzyme intermediates and catalytic mechanism

The most important common feature of the catalytic mechanism for peroxidases is that two spectrally distinct intermediates, commonly called compound I and compound II, are encountered during the enzymatic oxidation of substrates by hydrogen peroxide or organic hydroperoxide or peroxy acids. For HRP, compound I (HRP-I) is formed by the addition of a stoichiometric amount of hydrogen peroxide to the native enzyme. Both oxidizing equivalents of the hydrogen peroxide are acquired by the enzyme during this step, thus the enzyme is formally at a +5 state. Compound I appears to be a ferryl porphyrin π-cation radical, wherein one of the oxidation equivalents is stored as the ferryl iron, Fe(IV), while the other equivalent is stored as a porphyrin-centered π-cation radical. Electron donating substrates reduce HRP-I to the native ferric enzyme in two steps via the formation of compound II (HRP-II), the second intermediate of the enzyme. HRP-II contains one oxidizing equivalent above the native ferric enzyme. Thus it is a formal +4 state of the enzyme.

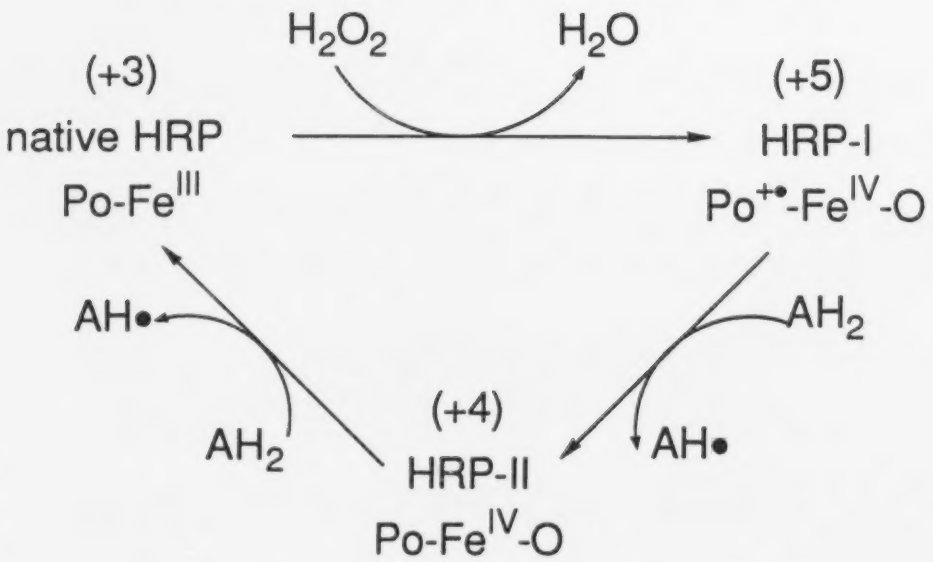
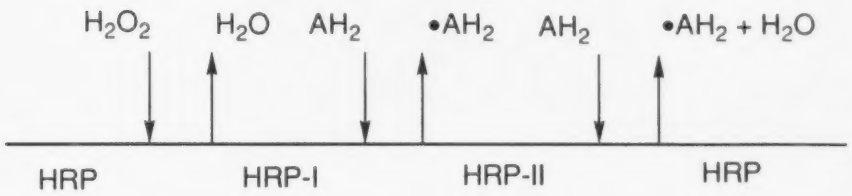


Figure 1.2. Normal catalytic cycle of HRP. AH_2 is a reducing substrate. Po represents the porphyrin ring of the heme.

The normal catalytic cycle for HRP is depicted in Fig. 1.2, which consists of a single two-electron oxidation of the enzyme followed by two single-electron reductions. The enzyme cycle can be represented as a modified form of ping-pong kinetics in which one molecule of oxidizing substrate (H_2O_2) and two molecules of reducing substrate (AH_2) are involved:



This leads to the overall reaction:

$$H_2O_2 + 2AH_2 = 2\cdot AH + 2H_2O$$

The free radical product has several fates, depending upon its structure and the environment. It may dimerize, react with another substrate molecule, or attack another species causing cooxidation. It may also reduce dioxygen, if present, to superoxide or it may be scavenged by dioxygen to form a peroxyl radical.

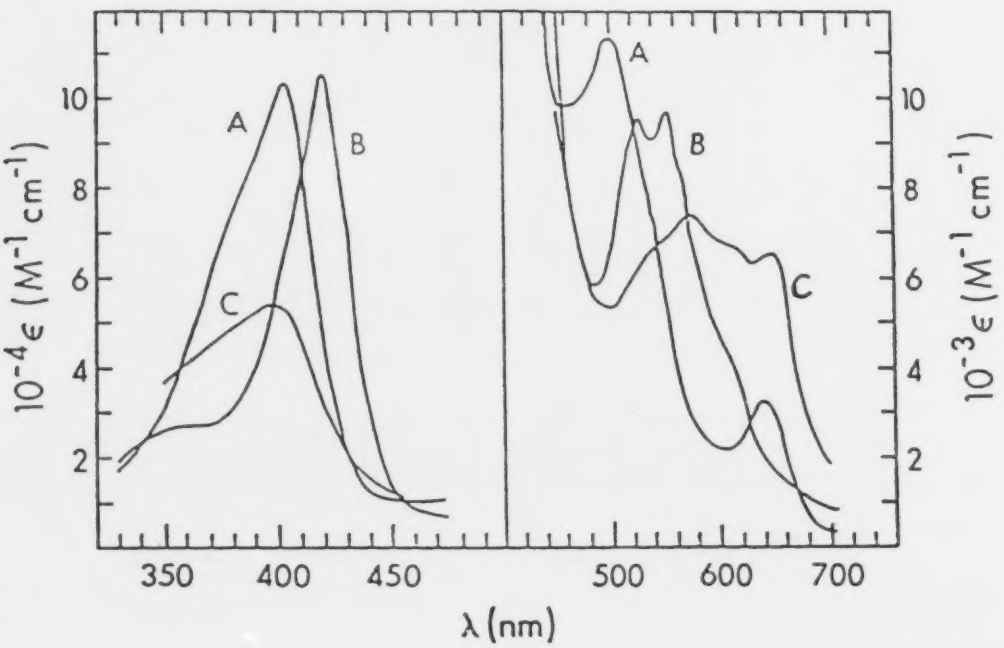


Figure 1.3. Electronic absorption spectra of native HRP , HRP-I, and HRP-II. A, native HRP; B, HRP-II; C, HRP-I.

The three enzyme species of HRP, i.e., native HRP, HRP-I, and HRP-II, are easily distinguished by their Soret and visible spectra, as shown in Fig.1.3.

One of the reasons why HRP is often chosen as a model of other peroxidases is that all the discovered members of the peroxidase family form similar one-and two-electron oxidized intermediates.

1.2 ENZYME-MEDIATED CARCINOGENESIS OF AROMATIC AMINES

1.2.1 Carcinogenesis of aromatic amines

A central tenet of cancer research is that tumors arise from cells that have undergone a permanent heritable change in their genetic material. Although a number of mechanisms can be envisaged to explain the origin of these heritable genetic changes, a dominant theme in present-day carcinogenesis is that they arise from the interaction between chemical carcinogens and DNA. This belief is supported by several lines of evidence including the facts that (i) there is a correlation between carcinogenic potency and extents of binding of carcinogen to DNA (40); (ii) the metabolic pathway responsible for carcinogen binding to DNA is usually the pathway which

produces carcinogenically active metabolites (41,42); (iii) most carcinogens are also mutagens provided appropriate metabolic activation systems are present (43); and (iv) the transformed phenotype can be transferred from one cell to another by transfecting DNA from one cell to another (44).

Based on their mechanisms of action on DNA, chemical carcinogens can be loosely classified as "genotoxic", "epigenetic" or "foreign-body" carcinogens. Genotoxic carcinogens produce DNA damage through covalent binding (or, in some cases, through strand scission). Epigenetic carcinogens are those that do not damage DNA directly, but may act by a variety of not clearly defined extrachromosomal mechanisms such as peroxisome proliferation, production of endocrine imbalance, immuno-suppression, etc. (45). Foreign-body carcinogens are those which, by virtue of their critical molecular shape and size, induce carcinogenesis probably by disrupting intercellular homeostasis or by mechanically interfering with DNA conformational changes. A survey of over 3,000 chemical carcinogens provides a rough estimate that more than 3/4 of the chemical carcinogens are genotoxic (46-50). In accordance with the heuristic theory first proposed by the Millers (51) in the 1960's, virtually all genotoxic carcinogens produce, either spontaneously or after metabolic activation, electrophiles which react with nucleophilic sites in DNA through covalent binding. Most of the carcinogens require metabolic activation; hence they are often called "procarcinogens".

The terminal electrophilic metabolites responsible for covalent binding to DNA are termed "ultimate carcinogens", whereas the intermediate metabolites leading to the terminal metabolites are termed "proximate carcinogens".

Aromatic amines are valuable chemicals in many areas of industry and research, particularly as intermediates in the dyestuff and pharmaceutical industries. However, some aromatic amines and their derivatives are well-known cancer producing agents that are active both in experimental animals and humans (Fig. 1.4). The induction of tumors by aromatic amines was originally traced to occupational exposure. In 1895, the German physician Rehn noted an increased incidence of bladder cancer in persons involved in the manufacturing of aromatic amine based dyes (52). Subsequently, Case et al. (53) found a similar relationship among British dye and rubber workers, which they attributed to 2-naphthylamine and benzidine (Fig. 1.4). More recently, exposure to another aromatic amine, 4-aminobiphenyl (Fig. 1.4), which was used as an antioxidant in the manufacturing of rubber products, was found to lead to an increased incidence of bladder tumors (54).

It has been known that almost all carcinogenic aromatic amines are procarcinogens, that is, they are not directly electrophilic but are converted into electrophilic derivatives through metabolic activation.

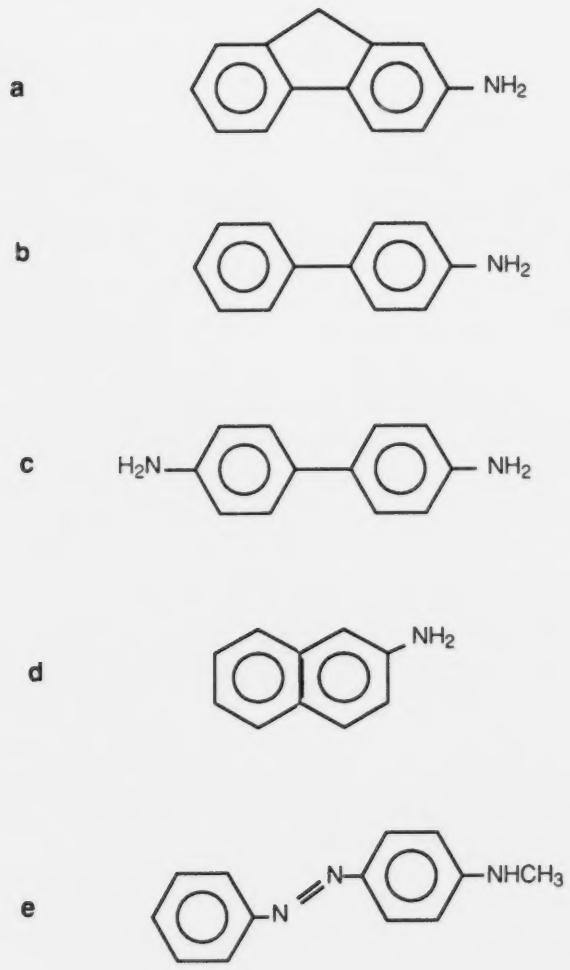


Figure 1.4. Representative carcinogenic aromatic amines.

a, 2-aminofluorene; b, 4-aminobiphenyl; c, benzidine;

d, 2-naphthylamine; e, N-methyl-4-aminoazobenzene.

1.2.2 Enzymatic activation of aromatic amines

The majority of chemical carcinogens including carcinogenic aromatic amines are activated by oxidative metabolism. As far as the initial step of oxidative activation is concerned, two major enzyme systems are involved : cytochrome P-450 dependent mixed-function oxidases, which catalyze the two-electron oxidation of arylamines to their N-hydroxy derivatives, and peroxidases, which catalyze the one-electron oxidation of arylamines to free radical species.

The activation of carcinogenic aromatic amines by enzyme systems in hepatic microsomes has been studied for many years (55,56). The enzyme systems involved can be divided into two groups: phase I and phase II (57). During phase I metabolism, one or more functional groups (such as hydroxyl) are introduced into the molecule. Phase II metabolism involves biosynthetic reactions in which the functional groups of phase I metabolites react with endogenous moieties (e.g., sulfate) to form ultimate carcinogenic products.

One of the most important phase I enzyme systems is a group of enzymes known as the cytochrome P-450 dependent mixed-function oxidases (MFO) or monooxygenases in which cytochrome P-450 serves as the oxidation-reduction component operational in bringing together substrate, oxygen, and reducing equivalents. In the monooxygenation of aromatic

amines, one atom of molecular oxygen is reduced to water while another is incorporated into the amino group, forming N-hydroxy derivatives of amines. Since the electrons involved in the reduction of cytochrome P-450 are derived from NADPH, the overall reaction can be written in the following way (Ar represents the aryl group):

$$ArNH_2 + O_2 + NADPH + H^+ \longrightarrow ArNHOH + H_2O + NADP^+$$

This is often called two-electron oxidative activation of aromatic amines. The N-hydroxy derivatives require phase II metabolism to form the electrophilic reactant which is the ultimate carcinogen. Two major metabolic pathways for N-hydroxy derivatives of aromatic amines have been established: sulfation and acetylation (58-61). The former is catalyzed by sulfotransferase with the cofactor of 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The latter is catalyzed by N-acetyltransferase, N,O-acyl transferase or O-acetyltransferase with the cofactor of acetyl coenzyme A (AcCoA).

Thus, the overall reaction sequence for the two-electron oxidative activation involves (i) initial N-hydroxylation, (ii) sulfation, acetylation, or N-acetylation accompanied by N,O-acyl transferase, and (iii) departure of sulfate or acetoxy group (Fig. 1.5). The products, arylnitrenium ions, are reactive electrophiles and ultimate carcinogens.

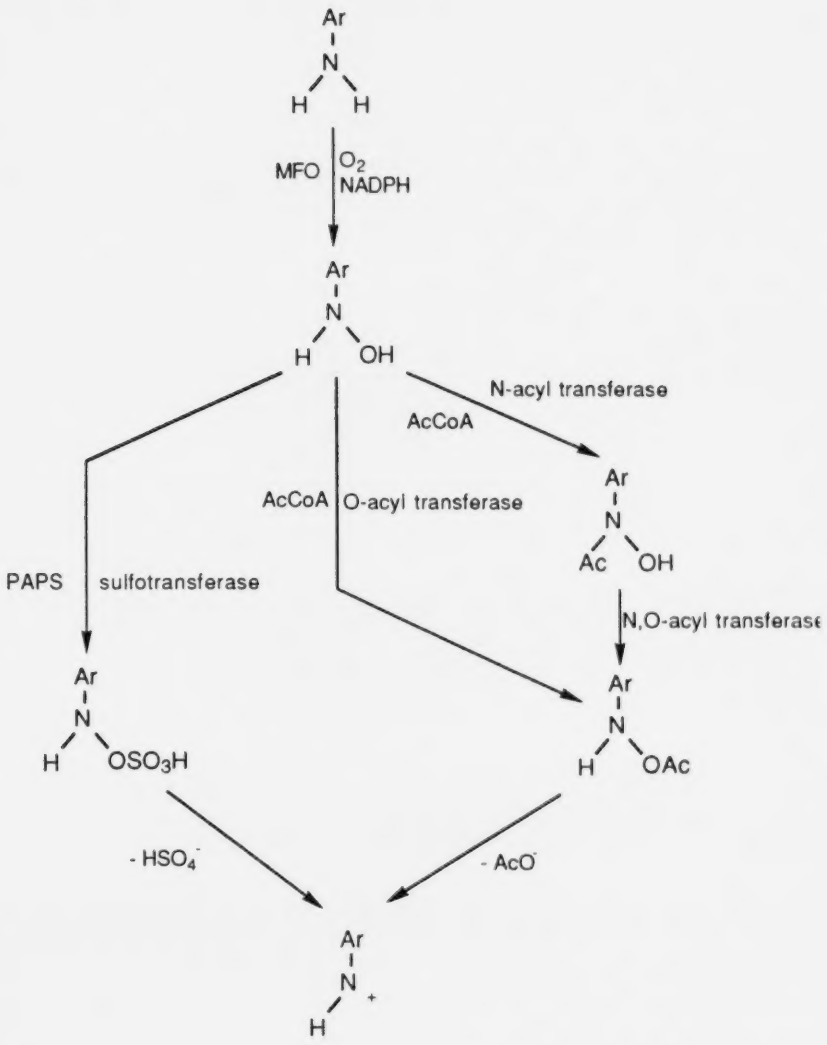
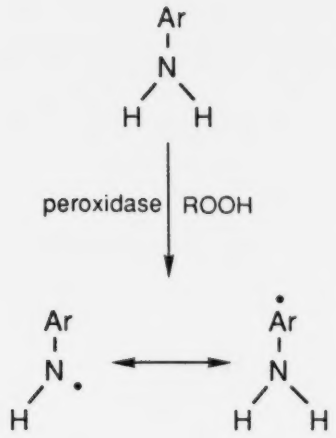


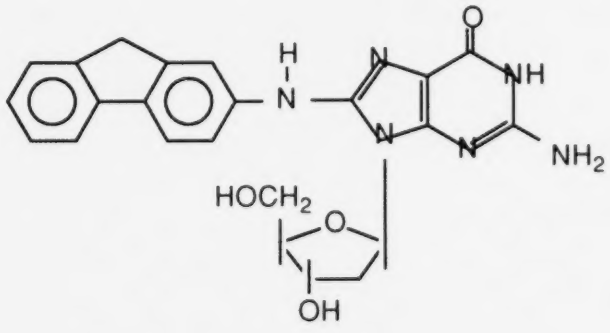
Figure 1.5. Two-electron oxidative activation of aromatic amines involving cytochrome P-450 dependent mixed-function oxidase (MFO) system.

In contrast to the two-electron oxidative metabolism involving cytochrome P-450 system, the one-electron oxidative activation of aromatic amines by peroxidases was not realized until recent years. A variety of chemical carcinogens including aromatic amines have been shown to be activated by prostaglandin H synthase (PHS) (62). As mentioned above, PHS consists of two components: a cyclooxygenase which catalyzes cyclization of arachidonic acid to yield PGG_2, and a peroxidase which converts PGG_2 to PGH_2 by one-electron reduction. One-electron oxidation of xenobiotics such as aromatic amines takes place at the peroxidase-catalyzed step through cooxidation reaction. Besides PHS, a number of extra-hepatic tissues, which have little or no MFO activity, contain peroxidase activities different from PHS. Examples include lactoperoxidase in the zymbal gland (63), an estrogen-dependent peroxidase in rat uterus (64), a lipoxygenase peroxidase in rat mammary gland (65). and a myeloperoxidase in leucocytes (66). The extra-hepatic tissues of these organs which are usually low in MFO activity are targets for certain cancer-causing arylamines suggesting that peroxidases are an alternative system for carcinogen activation.

The proposed ultimate carcinogenic metabolite in peroxidase-mediated metabolism of an aromatic amine is a nitrogen-centered free radical, which is the primary product of one-electron oxidation of the amine (67):



It is suggested that these electrophilic species, arylnitrenium ions or free radicals are reactive toward cellular DNA and capable of forming DNA adducts *in vivo* . Further studies indicate that most adducts are formed by covalent binding of the amine metabolite to C-8 of deoxyguanosine (68). In the case of 2-aminofluorene, for instance, the major adduct is N-(deoxyguanosin-8-yl)-2-aminofluorene:



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CHAPTER TWO

OXIDATION OF SUBSTITUTED ANILINES BY HRP-II*

2.1 SUMMARY

The kinetics of oxidation of eight monosubstituted anilines catalyzed by horseradish peroxidase compound II has been studied at pH 7.00 and pH 7.60. With p-toluidine the rates of oxidation by compound II have been measured at 21 pH's between pH 3.60 and pH 10.25. The rate-pH profile indicates that an acidic form of compound II and the electrically neutral, unprotonated form of p-toluidine are reactive. The correlation of rate constants for the substituted anilines with the substituent constant σ in the Hammett equation suggests that the aromatic amine donates an electron to compound II in the rate-controlling step and loses a proton simultaneously. The value of ρ, the susceptibility factor in the Hammett equation, is -6.0 ± 0.7. The reactivity of anilines with HRP-II observed in this study is lower than that of anilines with HRP-I observed previously, although the value of ρ is the same within experimental error [D. Job and H. B. Dunford. *Eur. J. Biochem.* **66**, 607 (1976)]. The difference in reactivity is explained by the relative complexities of compound I and compound II reactions.

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Abbreviations : HRP, horseradish peroxidase (EC 1. 11. 1. 7 donor-H_2O_2 oxidoreductase) ; HRP-I and HRP-II are compounds I and II of HRP.

2.2 INTRODUCTION

The reaction of aromatic amines (and other organic substrates) with horseradish peroxidase may be summarized as follows:

[2.1] $$HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O$$

[2.2] $$HRP\text{-}I + AH_2 \longrightarrow HRP\text{-}II + \cdot AH$$

[2.3] $$HRP\text{-}II + AH_2 \longrightarrow HRP + \cdot AH + H_2O$$

Horseradish peroxidase and its two oxidized intermediates, compound I and compound II, are represented by HRP, HRP-I and HRP-II respectively, the aromatic amines by AH_2 and their free radical product by ·AH. However, the general scheme above does not provide the details of events taking place on either the enzyme or the substrate during the oxidation process. Therefore a number of separate investigations have been conducted on HRP-catalyzed oxidation of various substrates in order to acquire better understanding and potential applications of the peroxidatic action on specific substrates. Examples can be found in (4-6).

The enzymatic oxidation of aromatic amines plays a significant role in many biotransformation processes such as chemical carcinogenesis and drug metabolism (7-9). Horseradish peroxidase in the presence of hydrogen

peroxide is a useful tool for modeling the extra-hepatic biooxidation of xenobiotics (10,11).

Substituent electronic effects are often studied as a probe into the reaction mechanism of aromatic compounds. The Hammett equation provides a general description of substituent effects upon reaction rates and equilibria of aromatic molecules (12):

[2.4] $$\log (k_X / k_H) = \rho\sigma$$

where k_X and k_H denote rate or equilibrium constants for reactions of the substituted and the unsubstituted compounds respectively; σ is the substituent constant, determined by the nature and the position of the substituent, and ρ is the reaction constant , also known as the susceptibility factor, which depends on the features and the conditions of the reaction. A modified form of the Hammett equation with σ replaced by a different substituent constant σ^+ is known as the Brown-Okamoto equation. It can correlate reactions deviating from the Hammett equation because of the generation of positive charge capable of resonance interaction with a substituent (13). A linear correlation by the Hammett relationship is an indication that a single mechanism is operating through the reaction series.

The Hammett $\rho\sigma$ correlation was previously established in the oxidation of substituted anilines by compound I of HRP. Compound I and compound II are two major intermediates in peroxidase reactions. It is then interesting to see whether the reactions between compound II of HRP and substituted anilines are also correlated by the Hammett equation, and if they are, how the correlation reflects the different structure and reactivity of compound II from those of compound I.

In this study, eight para or meta monosubstituted anilines were chosen to react with compound II of horseradish peroxidase. The rate-pH profile for one aromatic amine, p-toluidine, is also obtained. The Hammett relationship is employed to correlate the kinetic behaviors of the substituted anilines. The different susceptibility factor obtained in this study compared to that of anilines reacting with compound I of HRP is discussed.

2.3 EXPERIMENTAL

HRP, grade I, purchased from Boehringer-Mannheim as an ammonium sulfate precipitate, was dialyzed against water deionized in a Milli-Q water purification system. After the dialysis the RZ, the ratio of absorbance at 403 to 280 nm, was 3.28. The concentration of HRP was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} (14). Hydrogen peroxide was obtained as a 30% solution from Fisher.

The concentration of diluted H_2O_2 stock solution was determined by peroxidase assay as described elsewhere (15). All the aromatic amines, obtained from various sources (p-aminophenol and p-toluidine from BDH, p-phenetidine and m-phenetidine from Eastman, aniline from Mallinckrodt,and 3-aminoacetophenone, 4-aminobenzoic acid and sulfanilic acid from Aldrich), were of reagent grade and were used without further purification.

For each substrate, a stock solution of around 10 mM concentration was prepared by dissolving a weighed amount of the compound in volumetric flasks with purified water. All substrate solutions were stored in dark brown bottles to prevent photochemical reactions. The substrate solution for each experiment was then made by diluting the stock solution to the desired concentration with an appropriate buffer immediately before the measurement.

All the buffer solutions had an ionic strength of 0.11M, adjusted by adding 0.1M KNO_3 as an inert salt. Acetate buffer was used between pH 3.60 and 5.39, phosphate buffer between pH 5.58 and 7.60, Tris buffer between pH 8.05 and 9.04, and carbonate buffer between pH 9.30 and 10.25. All pH measurements were made with a Fisher Accumet Model 420 digital pH meter which was calibrated with standard buffers from Fisher.

Kinetic measurements for three substrates (3-aminoacetophenone, 4-aminobenzoic acid and sulfanilic acid) were performed on a Cary 219

spectrophotometer while the kinetic measurements for other substrates were made on a Photal (formerly Union Giken) stopped-flow spectrophotometer Model RA-601. The 1-cm observation cells of both spectrophotometers were thermostated at 25.0 °C for all the measurements.

The enzyme concentration for all the kinetic measurements was 1 μM, prepared with appropriate buffers. At least a 10-fold excess of substrates was used to maintain pseudo-first-order conditions.

HRP-II solution was freshly prepared prior to the experiments by addition of one equivalent of H_2O_2 and one equivalent of $K_4Fe(CN)_6$ to the native HRP solution weakly buffered at pH 9.0. No HRP-I was present because no change in absorbance was observed when the preparation of HRP-II was monitored at 411 nm, the isosbestic point between native HRP and HRP-II. HRP-II preparations were stable for the time span of all experiments. HRP-II reactions were monitored at 427 nm, where the maximum difference in absorbance between native HRP and HRP-II is expected.

For the stopped-flow experiments one drive syringe contained the HRP-II solution and the other was filled with the substrate solution. Between 5 and 8 traces were collected for each substrate concentration. The relative standard deviation was below 5% in most cases and never beyond 10%. For the measurements with the Cary spcetrophotometer, freshly prepared compound II solution was pipetted into the observation cell. The reference

cuvette was filled with purified water. The reaction was initiated by adding a calculated volume of the substrate strongly buffered at the desired pH into the cell containing HRP-II. The instrument was started immediately after the addition of the substrate. Measurements of relative absorbance at regular time intervals from the recorded trace followed by a nonlinear least square analysis on computer gave the observed pseudo-first-order rate constants.

2.4 RESULTS

With substrate in excess of HRP-II, pseudo-first-order kinetics was observed. One example is shown in the inset of Fig. 2.1. The corresponding differential rate expression is

$$[2.5] \qquad -\frac{d[\text{HRP-II}]}{dt} = k_{obs}\,[\text{HRP-II}]$$

where k_{obs} is the observed pseudo-first-order rate constant. The apparent second order rate constant, k_{app}, is related to k_{obs} by the equation

$$[2.6] \qquad k_{obs} = k_{app}\,[AH_2]$$

where $[AH_2]$ is the substrate concentration. Values of k_{app} were then obtained from the slopes of plots of k_{obs} versus $[AH_2]$, as shown by an example in Fig. 2.1.

The plot of the log k_{app} versus pH is shown in Fig. 2.2 for the oxidation of p-toluidine by HRP-II. It appears from the plot that two ionizations are occurring which affect the kinetics of the reaction.

The Hammett plot of log (k_x / k_H) versus σ (see eq. [2.4]) is shown in Fig. 2.3. The values of the second order rate constants at both pH 7.60 and pH 7.00, along with the Hammett σ values of different substrates are listed in Table 2.1. Values of k_{app} used to construct the plot are the averages of those obtained at the two pH's. The purpose of conducting the measurements at two different pH's is to confirm that k_{app} values for the Hammett plot were measured in such a region where the substituent effect, instead of pH effect, is predominant. As can be seen from the table, the k_{app} values at two pH's are the same within the experimental error.

The slope of the Hammett plot in Fig. 2.3 is equal to ρ in the Hammett equation. The value of ρ is -6.0 ± 0.7.

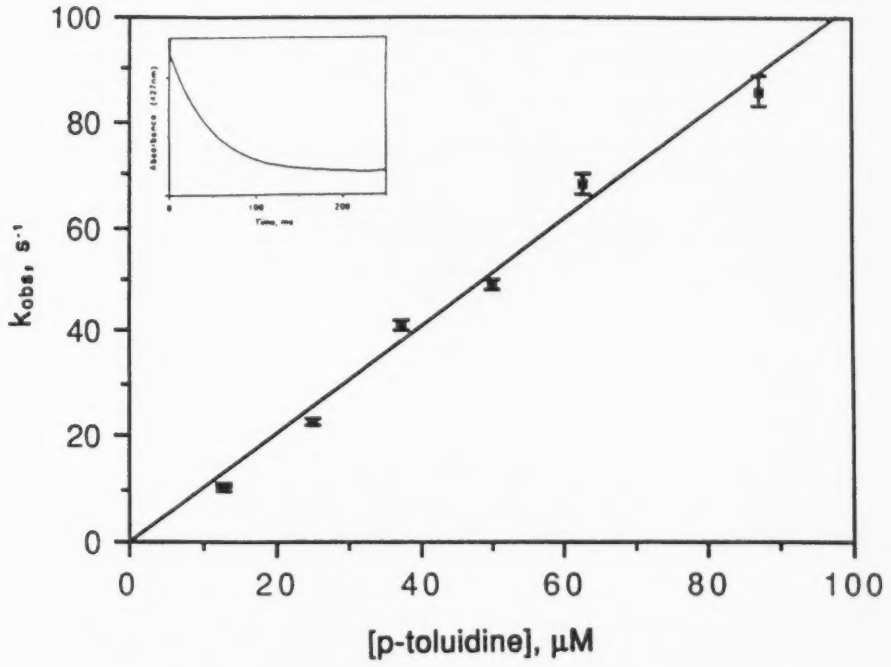


Figure 2.1. Plot of k_{obs} versus p-toluidine concentration for the oxidation of p-toluidine by horseradish peroxidase compound II. The reactions were carried out in phosphate buffer, ionic strength 0.11M; pH 7.60; [HRP-II], 1.0 μM; temperature, 25.0^{0}C. The inset shows an example of first order exponential trace (25 μM p-toluidine) from which k_{obs} was obtained.

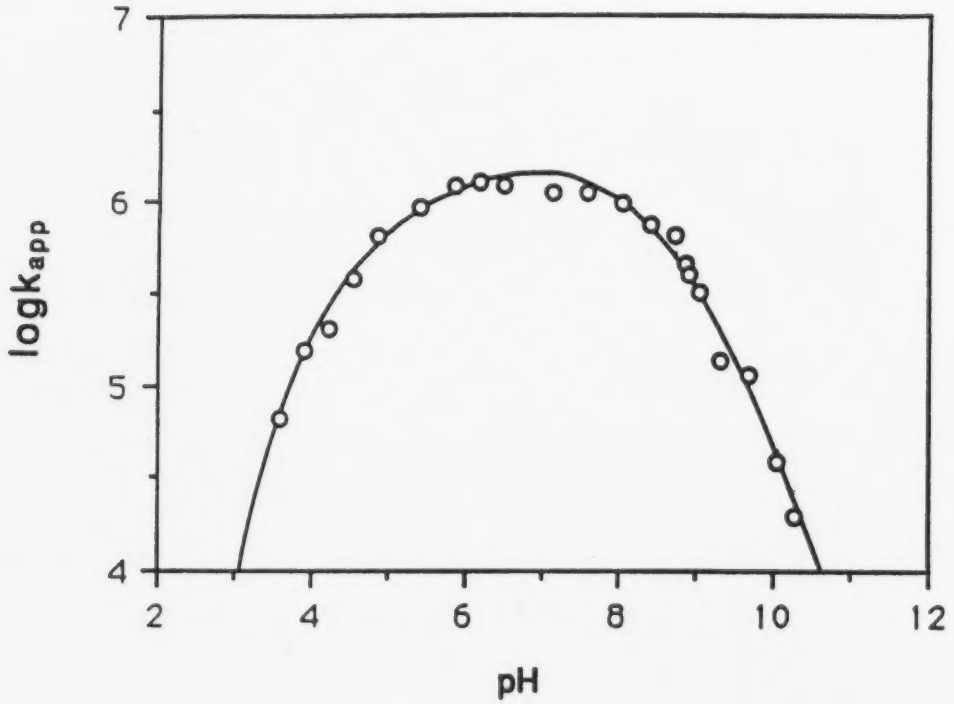


Figure 2.2. The rate-pH profile for the reaction of horseradish peroxidase compound II with p-toluidine at 25.0^{0}C and ionic strength 0.11M. The curve was computed on the basis of best-fit parameters obtained by a nonlinear least square analysis of the data with eq. [2.8].

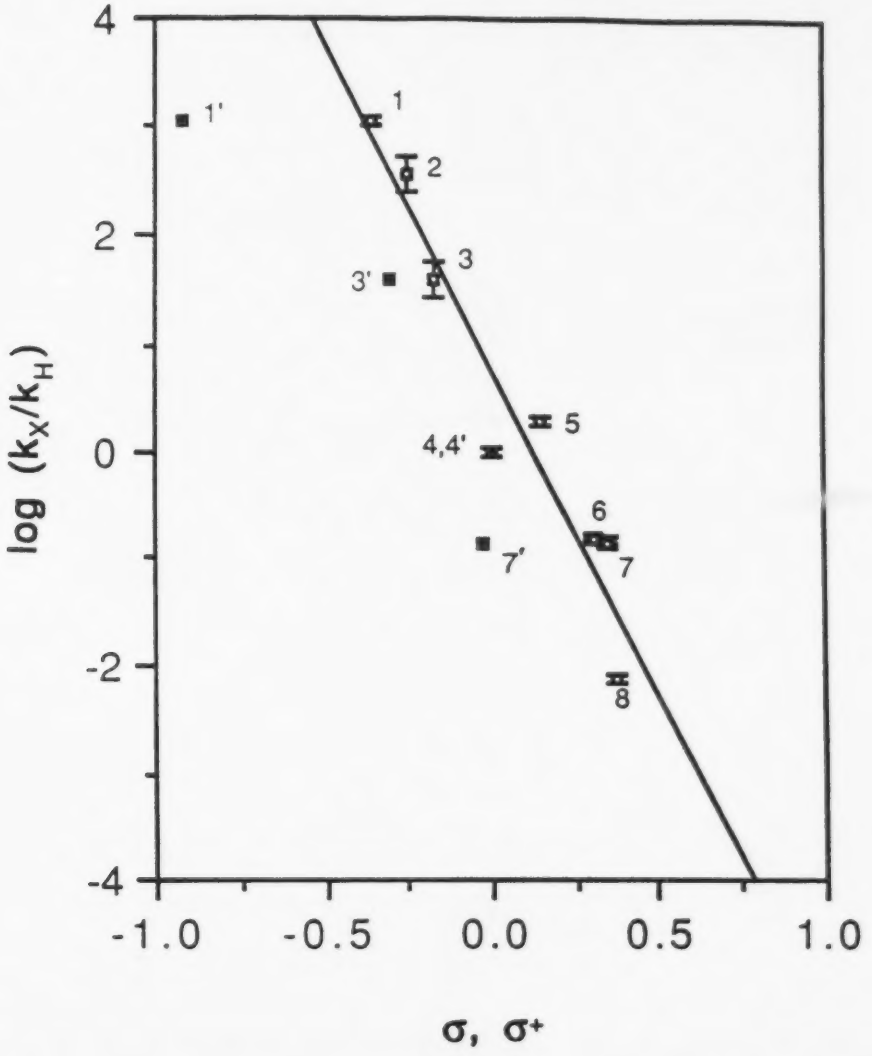


Figure 2.3. Hammett plot (o) and Brown-Okamoto (•) plot for the oxidation of various p- and m-substituted anilines by horseradish peroxidase compound II. k_X is the rate constant for the substituted aniline and k_H for aniline. The σ values are those listed in Table 2.1 and the σ^+ values are those obtained by Brown and Okamoto (13). Substituent groups: (1,1') p-OH; (2) p-OC_2H_5; (3,3') p-CH_3; (4,4') H; (5) m-OC_2H_5; (6) m-$COCH_3$; (7,7') p-COO^-; (8) p-SO_3^-.

TABLE 2.1. Effect of substituents on oxidation rate constants of aromatic amines by horseradish peroxidase compound II

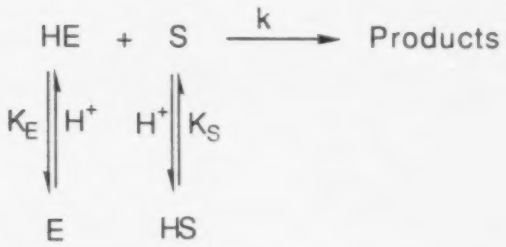
Substituent	k_{app} ($M^{-1}s^{-1}$)		σ	
	pH 7.60	pH 7.00	Value	Reference
p-OH[a]	$(3.5\pm0.3)\times10^{7}$	$(3.1\pm0.4)\times10^{7}$	-0.357	(16)
p-OC_2H_5	$(1.2\pm0.4)\times10^{7}$	$(1.0\pm0.3)\times10^{7}$	-0.25	(17)
p-CH_3	$(1.1\pm0.3)\times10^{6}$	$(1.2\pm0.4)\times10^{6}$	-0.17	(12)
H	$(2.8\pm0.1)\times10^{4}$	$(2.9\pm0.3)\times10^{4}$	0	
m-OC_2H_5	$(5.7\pm0.5)\times10^{4}$	$(5.5\pm0.2)\times10^{4}$	0.15	(17)
m-$COCH_3$	$(4.1\pm0.2)\times10^{3}$	$(4.4\pm0.1)\times10^{3}$	0.306	(12)
p-COO^-	$(4.0\pm0.1)\times10^{3}$	$(3.8\pm0.1)\times10^{3}$	0.35	(16)
p-SO_3^-	$(2.0\pm0.2)\times10^{2}$	$(2.3\pm0.2)\times10^{2}$	0.38	(16)

[a] Value (pH 7.60) reported in (22).

2.5 DISCUSSION

The simplest mechanism which will account for the experimental data is one in which the neutral unprotonated amine reacts with an acidic form of the enzyme :

[2.7]



The k_{app}-pH relationship corresponding to eq. [2.7] can be described as follows:

[2.8]

$$k_{app} = \frac{k}{\left[1 + \frac{K_E}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_S}\right]}$$

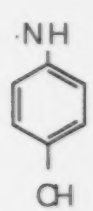
Using nonlinear least-square analysis, the experimental log k_{app} - pH data points shown in Fig. 2.2 were fit to the eq. [2.8]. The best-fit parameters are k = $(1.2 \pm 0.1) \times 10^6\ M^{-1}s^{-1}$; $K_E = (2.8 \pm 0.3) \times 10^{-9}$ M, $pK_E = 8.6 \pm 0.1$; $K_S = (1.5 \pm 0.2) \times 10^{-5}$ M, $pK_S = 4.8 \pm 0.1$. The curve computed on the basis of the best-fit

parameters is also shown in Fig. 2.2. Previous studies on the HRP-II reactions with different substrates have proved the existence of an acid-base group on the enzyme with a pK_a value of 8.6 so that our present results are in excellent agreement (5). The pK_{BH^+} of p-methyl anilinium ion (protonated p-toluidine) was reported to be 5.08 in water at 25^0 C (18), which can be corrected for this study by considering ionic strength in terms of the extended Debye-Hückel equation (19). The pK_{BH^+} so corrected is 4.97. Similarly there is a good agreement between our pK_S value computed from the kinetic results and the literature value for the ionization of protonated p-toluidine.

The adherence to the Hammett equation for para or meta monosubstituted anilines, as can be seen from Fig. 2.3, shows that all of the anilines react with compound II by the same mechanism. One conclusion can be drawn immediately, that is, the neutral forms of the anilines are reacting in all the aniline oxidations by compound II. A negative slope of the Hammett plot indicates that electron-donating groups, as we expected, facilitate the oxidation of anilines. This suggests that the removal of one electron from a substrate is the rate-controlling step in the oxidation of anilines. The loss of one electron from a substrate is accompanied by the simultaneous loss of a proton, so that a positive charge at the reaction site is not formed. The experimental data are better correlated by the Hammett $\rho\sigma$ relationship, instead of the Brown-Okamoto $\rho\sigma^+$ relationship (the correlation coefficients for

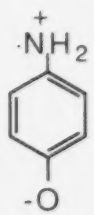
the two cases are 0.931 and 0.887 respectively). The plot of rate constants against σ^+ values can be seen in Fig. 2.3. Unfortunately some of the relevant σ^+ values are not available in the literature. A mechanism similar to ours has been suggested by different authors (20-23).

There have been discussions about the p-aminophenol oxidation by HRP as to whether it reacts as a substituted aniline or as a substituted phenol (21), in other words, whether the one electron oxidation and proton abstraction occur from the amino group, yielding free radical **1** or from the hydroxyl group, yielding free radical **2**.



1

2



3

Though **2** was cited as the oxidation product (24), there is evidence suggesting the oxidation on the amino group. In the oxidation by compound I, the rate constant for p-aminophenol seems to fit the Hammett plot for the aniline series. However, this could result from the oxidation on the hydroxyl group being retarded by partial diffusion control (21). Later studies on the reactions between phenols and compound II showed the obvious deviation of p-aminophenol from the Hammett plot of the phenol series, favoring the

possibility of p-aminophenol reacting as a substituted aniline (22). An ESR study of HRP-catalyzed oxidation of p-aminophenol performed under alkaline conditions demonstrated that the one electron oxidation product is a zwitterion radical **3** with the electron removed from the amino group (25). The fit of the data for p-aminophenol to the Hammett plot for anilines in this study also suggests that the removal of the electron and the proton takes place on the amino group instead of the hydroxyl group. This is interesting considering the fact that the reactivity of anilines is generally lower than that of phenols.

Compared with the ρ value of -7.0 ± 0.7 (21, standard deviation is calculated based on the original data) for the reaction of anilines with compound I, the ρ value obtained in this study for the reaction of anilines with compound II is -6.0 ± 0.7. Thus the substituent susceptibility of compound II reactions is the same within experimental error as that observed for the corresponding compound I reactions. This is in contrast to the results for phenols where ρ is -6.9 ± 0.5 for compound I (21, standard deviation is calculated based on the original data) and -4.6 ± 0.5 for compound II (22). In an independent work, published since this manuscript was originally submitted, a ρ value of -5.75 was obtained for substituted anilines reacting with HRP-II, in close agreement with our results presented here (23). In their work mainly different substituted anilines were used. As another comparison, the rate constants of anilines reacting with HRP-II obtained in this study are

about one order of magnitude lower than those of anilines reacting with HRP-I (21). A proposed mechanism for anilines reacting with both compound I and compound II is depicted in Fig. 2.4. It is widely accepted that compound I is a porphyrin π-cation radical (26). During the oxidation of anilines by compound I, the aromatic electron-deficient compound I acts as the direct target for an electron donated from amines (27,28). While in the case of compound II reactions, in addition to the transfer of an electron to iron(IV), two protons are also transferred to the ferryl oxygen forming a water molecule which leaves from the inner coordination sphere of the iron (29). This comparison indicates that the compound II reduction, which involves more bond-breaking and bond-formation steps, is a more complicated chemical process than compound I reduction (29). This explains the order-of-magnitude lower reactivity of anilines with HRP-II compared to HRP-I.

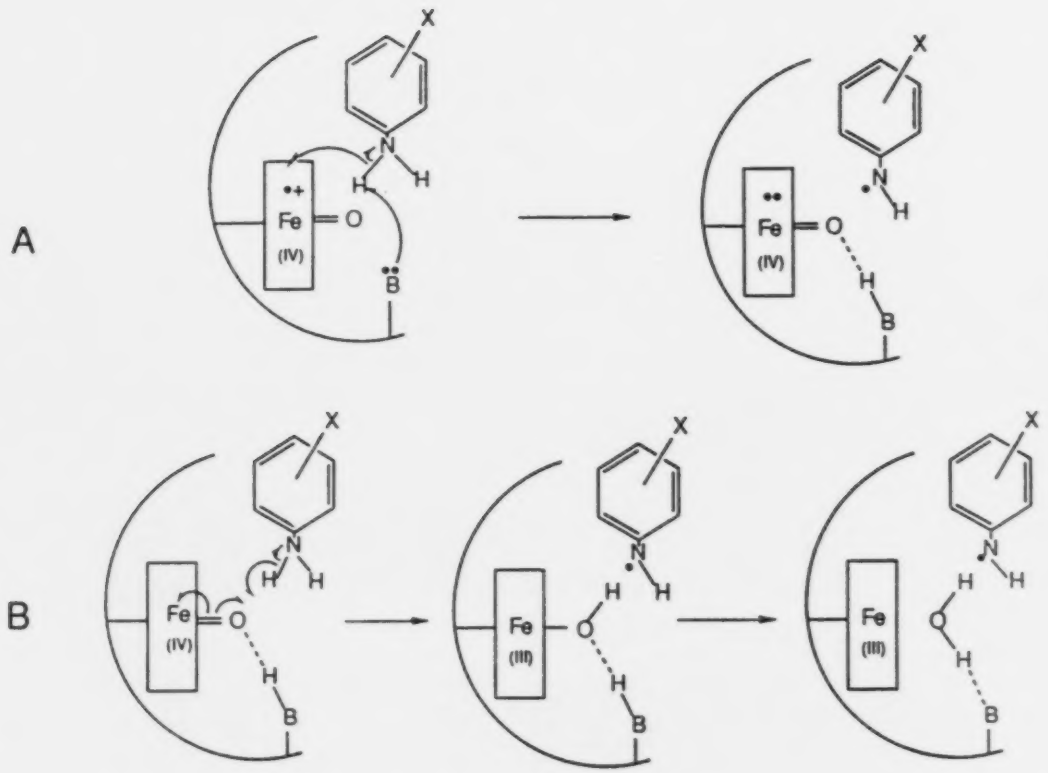


Figure 2.4. Schematic representation of relative complexities of compound I and compound II reactions. (A) Relatively simple reaction of compound I with anilines. An electron is transferred to the porphyrin ring (represented by the rectangle) neutralizing the π-cation radical of compound I. (B) Relatively complicated reaction of compound II with anilines. An electron is transferred to iron (IV) and two protons are transferred to the ferryl oxygen atom forming a water molecule as a leaving group.

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CHAPTER THREE

OXIDATION OF 2-AMINOFLUORENE BY HRP-I AND HRP-II*

3.1 SUMMARY

Horseradish peroxidase catalyzed oxidation of the model carcinogen 2-AF** was studied by rapid scan spectral analysis and stopped flow kinetic measurements. Our rapid scan spectral analysis of the changes in the enzyme oxidation states clearly indicates that the reactions between HRP intermediate compounds and 2-AF are one-electron redox processes, thus a free radical is necessarily the primary product. The reactions were investigated at pH's ranging from 3.40 to 10.00. The pH profiles of rate constants suggested that 2-AF is reactive toward HRP only when it is in its neutral form; one acid-base group of pKa~5 in HRP-I, and one of pKa~8.6 in HRP-II affect the kinetics of HRP catalyzed 2-AF oxidation. The intrinsic

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** Abbreviations : 2-AF, 2-aminofluorene; HRP, horseradish peroxidase (EC 1. 11. 1. 7 donor-H_2O_2 oxidoreductase) ; HRP-I is compound I of HRP and HP-I, P-I indicate different states of protonation of compound I; similarly HRP-II is compound II of HRP and HP-II, P-II differ by one proton; DMSO, dimethyl sulfoxide; ESR, electron spin resonance.

second order rate constants of 2-AF oxidation are $(1.1 \pm 0.5) \times 10^9$ $M^{-1} s^{-1}$ and $(4.8 \pm 0.4) \times 10^8$ $M^{-1} s^{-1}$ for HRP-I, the two values depending upon the state of protonation of HRP-I, and $(1.7 \pm 0.2) \times 10^8$ $M^{-1} s^{-1}$ for HRP-II. It is suggested that the rate-controlling step is formation of a substrate cation radical, which is stabilized by the extensive π-conjugation system of 2-AF. This is supported by the high reactivity of 4-aminobiphenyl, a similar compound, which does not fit the Hammett ρσ correlations established for other substituted anilines; these other anilines react by hydrogen atom transfer. Thus the extraordinarily rapid reactions between 2-AF and HRP compounds I and II are attributed to the strong aromacity of 2-AF.

3.2 INTRODUCTION

Of all the carcinogenic aromatic amines, 2-AF and its derivatives have a unique position because they have long been used as models in research on chemical carcinogenesis (1-4). The initial intended use of 2-AF as an insecticide never turned into commercial development because of its carcinogenicity. Though it is generally accepted that some eletrophilic metabolites of 2-AF and its derivatives are responsible for cancer-inducing DNA binding, the nature of the ultimate carcinogenic species and the mechanism of the formation of these species have been a focus of the research on chemical carcinogenesis for many years.

Most studies on the mechanism of activation of carcinogenic arylamines have been centered around the cytochrome P-450 dependent mixed function oxidase system, which is mainly located in the liver (5-8). It has long been believed that the oxidative activation of carcinogenic arylamines including 2-AF takes place in the liver; and it is characterized by an initial two-electron oxidation to N-hydroxyarylamines catalyzed by the cytochrome P-450 dependent mixed function oxidase system (9,10). However, evidence has accumulated that the administration of 2-AF to animals results in tumors not only in liver, but also in the mammary gland, urinary bladder, intestine, and sebaceous gland of the ear duct (11). Therefore, there must exist some other metabolic pathway(s) for 2-AF activation in extra-hepatic tissues where the level of cytochrome P-450 dependent monooxidase is much lower than that in the liver.

Since peroxidases are widely distributed in extra-hepatic tissues, it has been proposed that peroxidases may also be involved in the oxidative activation of carcinogenic arylamines (12-15). Horseradish peroxidase, often used as a model peroxidase in the studies of extra-hepatic oxidation of xenobiotics, has proved capable of activating 2-AF and its derivatives to mutagenic and electrophilic compounds which bind to macromolecules (16-18). The one-electron oxidation mechanism involving free radicals was proposed, which was mainly based on the analysis of metabolic products and

supported by the absence of N-hydroxy-2-AF (19). The ESR observation of the primary free radical was unsuccessful, which has left the one-electron oxidation hypothesis open to further experimental confirmation.

In this study we obtain more evidence on the one-electron oxidation mechanism obtained from the spectral analysis of enzyme oxidation state changes and investigated the kinetics of the oxidation of 2-AF by horseradish compounds I and II in a pH range between 3.40 and 10.00.

3.3 MATERIALS AND METHODS

Materials

2-AF and 4-aminobiphenyl were purchased from Aldrich Chemical Co. Stock solutions were prepared in 50% DMSO. DMSO, which proved inactive toward HRP and its intermediate compounds in our preliminary experiments, is necessary to solubilize 2-AF and 4-aminobiphenyl in the aqueous reation mixture.

HRP, grade I, was purchased from Boehringer-Mannheim as an ammonium sulfate suspension. The stock solution was prepared by dialyzing the suspension against deionized water and passing it through a Millipore filter prior to use. The purity number (A_{403nm}/A_{280nm}) of the HRP stock solution

is greater than 3.2. The concentration of HRP was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 $M^{-1}cm^{-1}$ (20).

H_2O_2 (30%) and $K_4Fe(CN)_6$ were obtained from Fisher. The concentration of $K_4Fe(CN)_6$ stock solution was determined by weight while that of H_2O_2 was determined by peroxidase assay (21).

All solutions were prepared using water purified by a Milli-Q Water Purification System from Millipore.

Preparation of HRP-I and HRP-II

All of the buffer solutions used to prepare HRP-I and HRP-II had an ionic strength of 0.11M, adjusted by adding potassium nitrate as an inert salt. Citrate buffer was used between pH 3.40 and 5.80, phosphate buffer between pH 6.20 and 8.00, and carbonate buffer between pH 8.50 and 10.00. A Fisher Accumet Model 420 digital pH meter was used for pH measurements.

HRP-I was prepared by the addition of one equivalent of H_2O_2 to the native enzyme in the buffer of the desired pH. A pH-jump method was employed in the case of HRP-II because of the difficulty of preparing pure compound II at neutral and acidic pH's. Thus stable HRP-II was obtained in the buffer of pH 10.00 by adding one equivalent of H_2O_2 and one equivalent of $K_4Fe(CN)_6$ to the native enzyme solution. In the experiments involving HRP-II,

the newly formed HRP-II in the buffer of pH 10.00 was placed in one of the cuvettes of the rapid scan spectrophotometer and 2-AF in the buffer of a lower pH in the other. The pH of the mixed solution was measured afterwards.

Determination of pK_{BH^+} for 2-AF

The pK_{BH^+} of 2-AF was determined on a Cary 219 spectrophotometer. Absorbance was measured at 286 nm, the wavelength at which the absorbance appeared most sensitive to the difference between the two ionization states. The system was investigated at 10 pH values from 2.60 to 7.40. Different pH's were achieved by using buffers in which the oxidation of 2-AF was actually investigated. One sample contains 15 μM 2-AF, 0.1 M potassium nitrate and buffer of ionic strength 0.01 M.

Rapid scan spectral analyses

Spectral analyses were made on a Photal (formerly Union Giken) Model RA-601 Rapid Reaction Analyzer which is equipped with a solid-state photodiode array. Spectral changes during reactions of HRP-I and HRP-II with 2-AF were monitored in the Soret region.

The rapid scan absorption spectra were recorded at pH 7.10. With the concentrations of HRP-I and the 2-AF being 0.5 μM and 1.0 μM, respectively, the accompanying spectral changes were recorded over the time interval 2-16

ms. The spectral changes during the reaction of HRP-II with 2-AF were recorded over the interval 2-16 ms, with the concentrations of the enzyme and the substrate being 1.0 μM and 2.0 μM, respectively.

Stopped-flow kinetic experiments

Stopped-flow experiments were also conducted on the Photal Rapid Reaction Analyzer of Model RA-601. Experimental curves were recorded and stored in the computer interfaced to the Rapid Reaction Analyzer.

Kinetic measurements were performed with the initial concentrations of both the enzyme and the substrate being 1.0 μM. The apparent second order rate constants were then obtained from the experimental curves through the computer program of non-linear least-square analysis.

Because of the similarity of 4-aminobiphenyl to 2-AF, and because the Hammett σ value is known for 4-aminobiphenyl ($\sigma_{p\text{-phenyl}}$ = -0.01) (22), we also measured the rate constants for the reactions of 4-aminobiphenyl with HRP-I and HRP-II at pH 7.0 and 25.0^{0}C.

The reaction of HRP-I was followed by monitoring the disappearance of HRP-I at 411 nm, the isosbestic point between native enzyme and compound II, and the reaction of HRP-II was monitored at 427 nm, the isosbestic point between native enzyme and HRP-I.

3.4 RESULTS

The pK_{BH^+} Value of 2-AF

The pH dependent absorbance of 2-AF at 286 nm is shown in Fig. 3.1, which was used to obtain the pK_{BH^+} value of 2-AF through the following equation:

[3.1] $$pK_{BH^+} = \log\left(\frac{A_B - A}{A - A_{BH^+}}\right) + pH$$

where A is the absorbance at a given pH, A_B and A_{BH^+} denote the absorbances of the neutral and the protonated forms of 2-AF, respectively. Data points were fitted to the equation using nonlinear least-square analysis. A pK_{BH^+} value of 4.57 ± 0.05 was obtained for 2-AF. The best fit curve is also shown in Fig. 3.1.

Oxidation of 2-AF by HRP-I

The spectral changes observed during the reaction indicate that HRP-I is first reduced to HRP-II, then converted to the native state (Fig. 3.2). The isosbestic point appearing at 411 nm is proof of an HRP-II-to-native HRP conversion with no detectable intermediates.

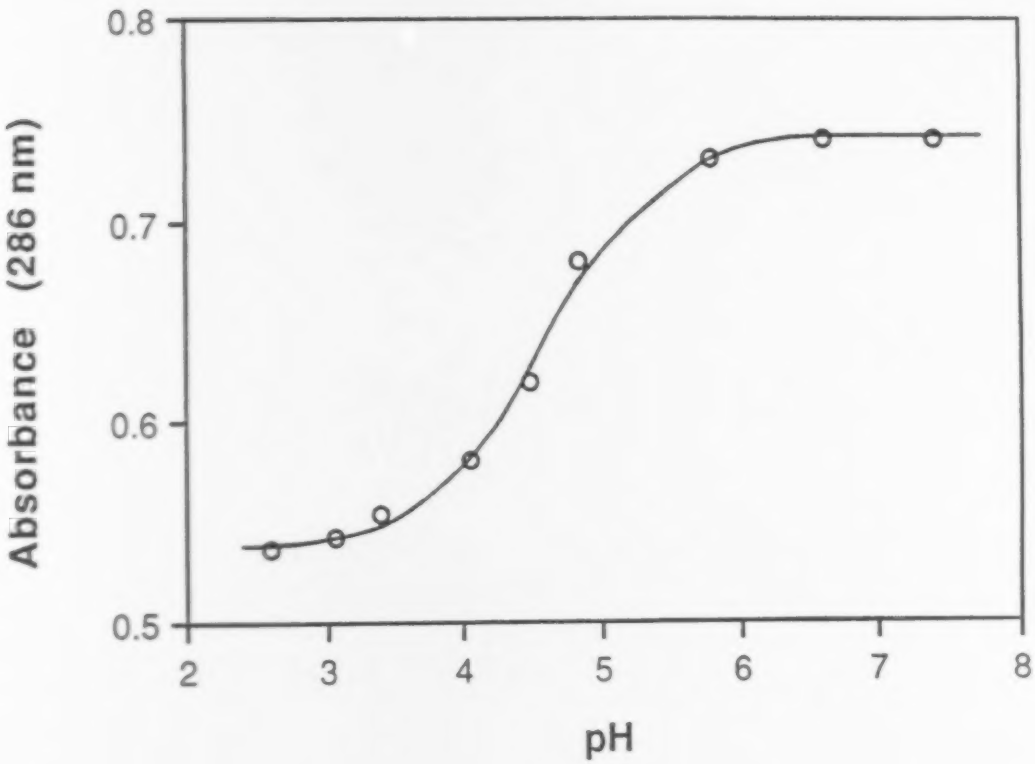


Figure 3.1. Plot of absorbance at 286 nm versus pH for 15 μM 2-AF in buffer solutions of total ionic strength 0.11 M at 25°C. The curve shows the nonlinear best fit to eq. [3.1]. A pK_{BH^+} value of 4.57 ± 0.05 was obtained.

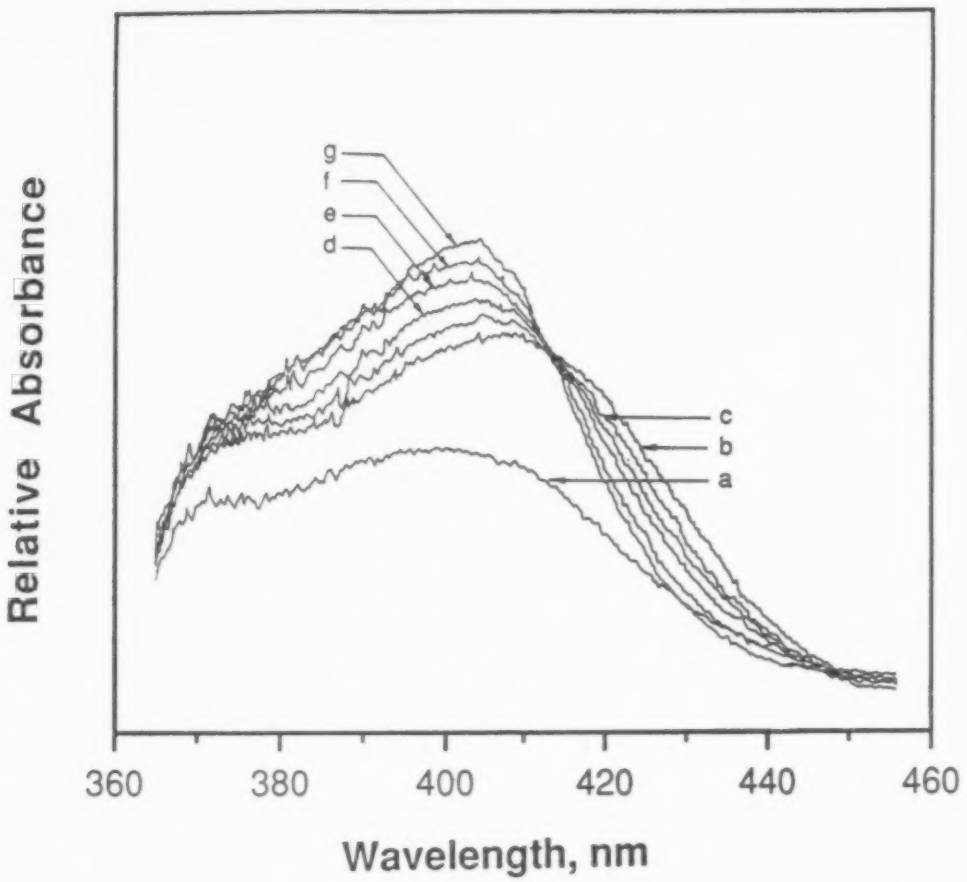


Figure 3.2. Rapid scan spectral changes observed during the reaction of HRP-I with 2-AF. The spectra were recorded at 25^0 C and pH 7.10. The final concentrations were 0.5 μM HRP-I and 1.0 μM 2-AF. HRP-I (a) was formed as described in Materials and Methods. Scans b, c, d, e, f, and g: 2, 4, 6, 8, 12, and 16 ms after mixing.

With the same initial concentrations of HRP-I and 2-AF, the time course of the reaction can be described by

[3.2][2] $$\frac{1}{[\text{HRP-I}]} - \frac{1}{[\text{HRP-I}]_0} = k_{1,app}\, t$$

where $[\text{HRP-I}]_0$ is the initial concentration of HRP-I, and $k_{1,app}$ is the apparent second order rate constant. Monitored at 411 nm on stopped-flow appratus, the disappearance of HRP-I resulted in hyperbolic traces of absorbance versus time. Such traces were then fitted using a nonlinear least square computer program to obtain $k_{1,app}$ values. One of the stopped-flow traces and its calculated fitting is shown in Fig. 3.3.

The acidity of the reaction medium affects the kinetics of the oxidation of 2-AF by HRP-I, as can be seen from Fig. 3.4. When the pH is low (<5), $k_{1,app}$ increases as pH increases. After a maximum is reached around pH 5, $k_{1,app}$ begins to decrease, and finally levels off at neutral and alkaline pH's.

A second order rate constant of $(5.0 \pm 0.2) \times 10^7\ M^{-1} s^{-1}$ for the reaction of 4-aminobiphenyl with HRP-I was obtained at pH 7.0.

Oxidation of 2-AF by HRP-II

The spectral data in Fig. 3.5 shows the conversion from HRP-II to native HRP in the reaction of the former species with 2-AF.Similar to the the

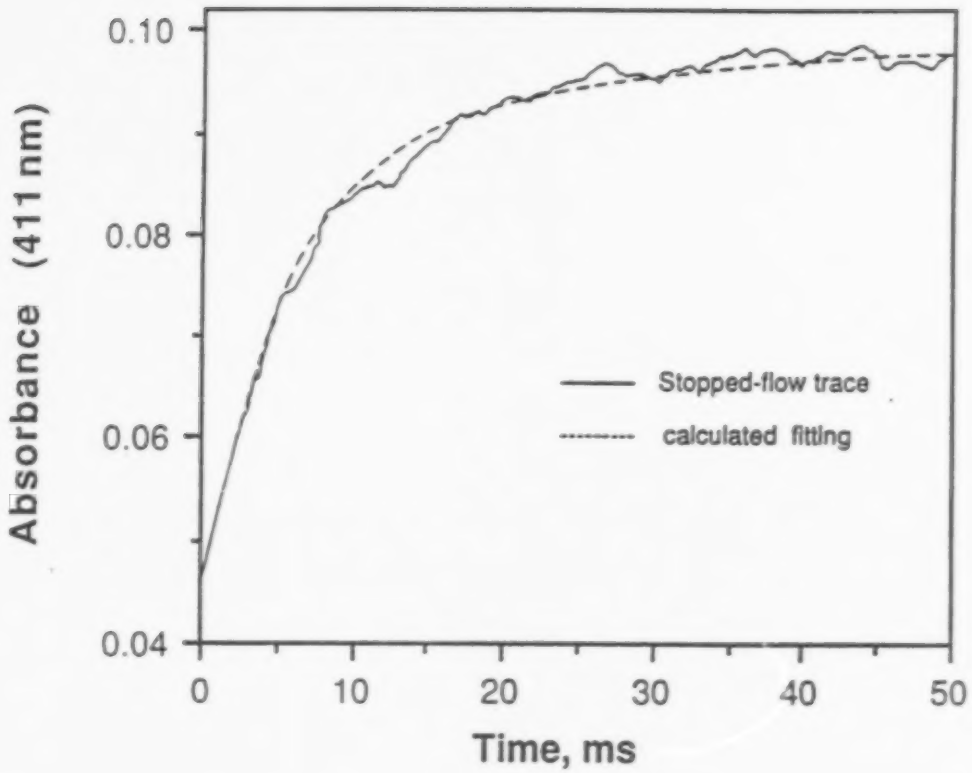


Figure 3.3. Stopped-flow trace of absorbance for the oxidation of 2-AF by HRP-I monitored at 411nm. The reaction was carried out at pH 7.10. Ionic strength, 0.11 M; $[HRP\text{-}I]_0$, 1 μM; $[2\text{-}AF]_0$, 1 μM; temperature, 25° C.

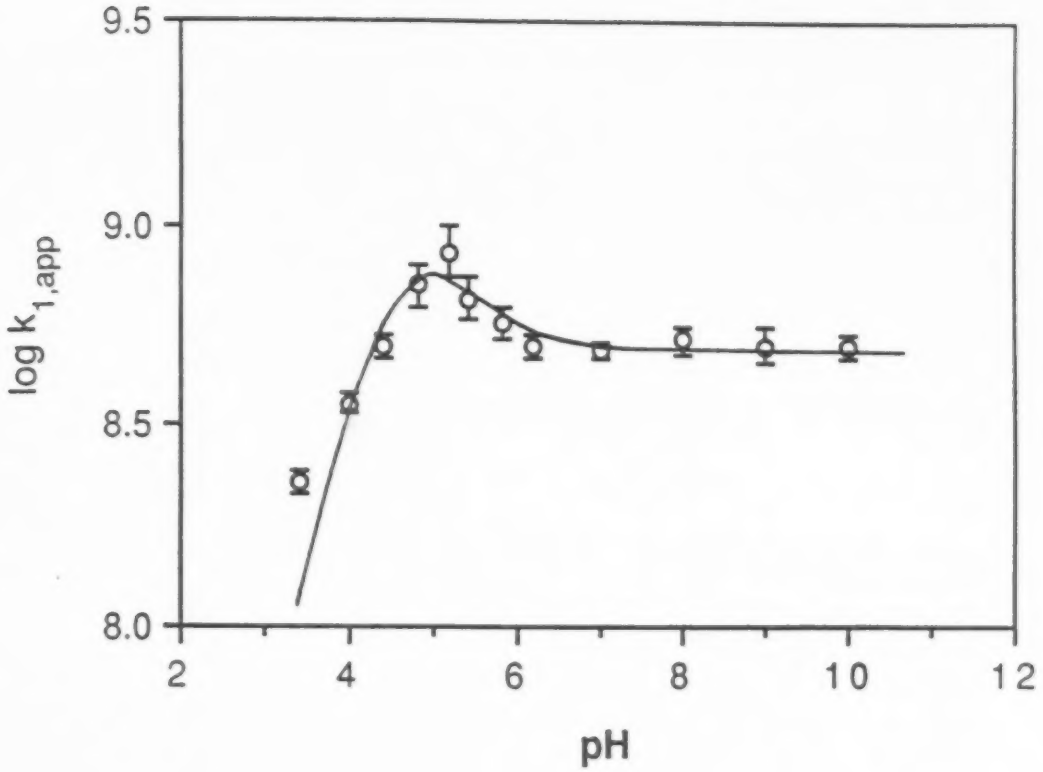


Figure 3.4. pH profile of second order rate constant for the reaction of HRP-I with 2-AF at 25°C and ionic strength 0.11 M. The curve was obtained through computation using eq. [3.4] on the basis of the best-fit parameters listed in Table 3.1.

TABLE 3.1. Parameters obtained from nonlinear least square analysis of pH profiles of second order rate constants of 2-AF reacting with HRP-I and HRP-II

Parameters	HRP-I + 2-AF	HRP-II + 2-AF
k_{int}	$(1.1 \pm 0.5) \times 10^9\ M^{-1} s^{-1}$ ($k_{1,int}$)	$(1.7 \pm 0.2) \times 10^8\ M^{-1} s^{-1}$
	$(4.8 \pm 0.4) \times 10^8\ M^{-1} s^{-1}$ ($k'_{1,int}$)	
pK_S	4.3 ± 0.6	4.5 ± 0.2
pK_E	5.3 ± 0.4	8.6 ± 0.2

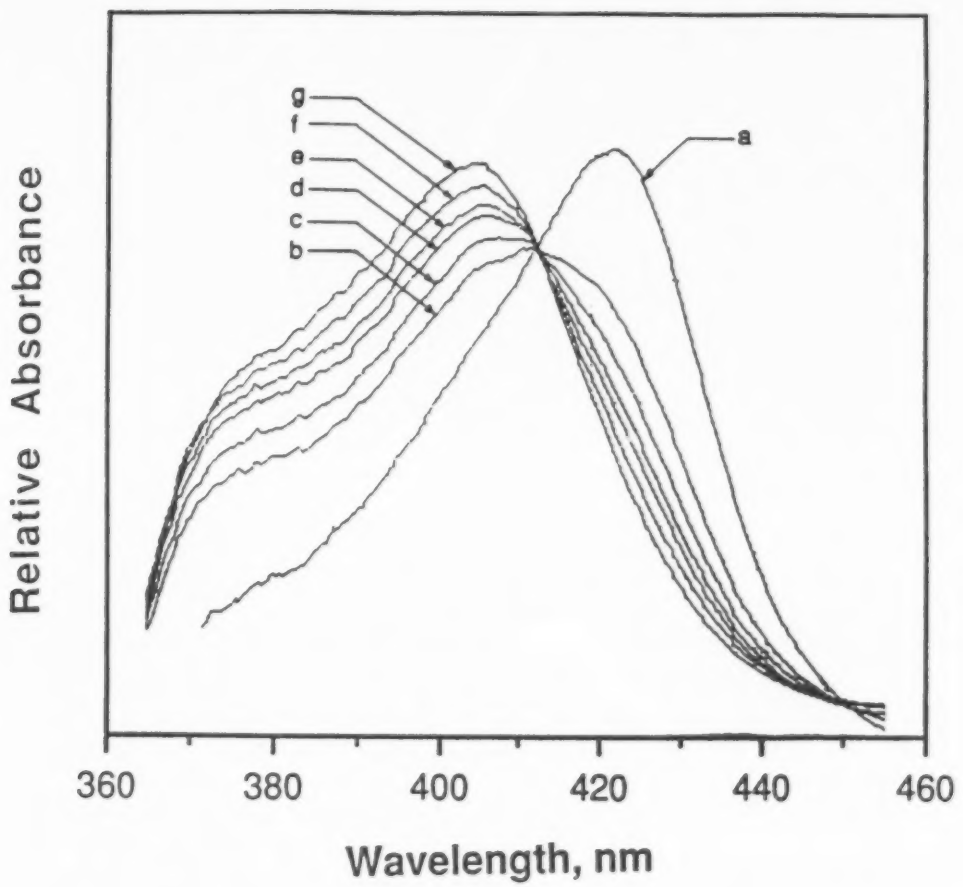


Figure 3.5. Rapid scan spectral changes observed during the reaction of HRP-II with 2-AF. The spectra were recorded at 25°C and pH 7.10. The final concentrations were 1.0 μM HRP-II and 2.0 μM 2-AF. HRP-II (a) was formed as described in Materials and Methods. Scans b, c, d, e, f, and g: 2, 4, 6, 8, 11, and 16 ms after mixing.

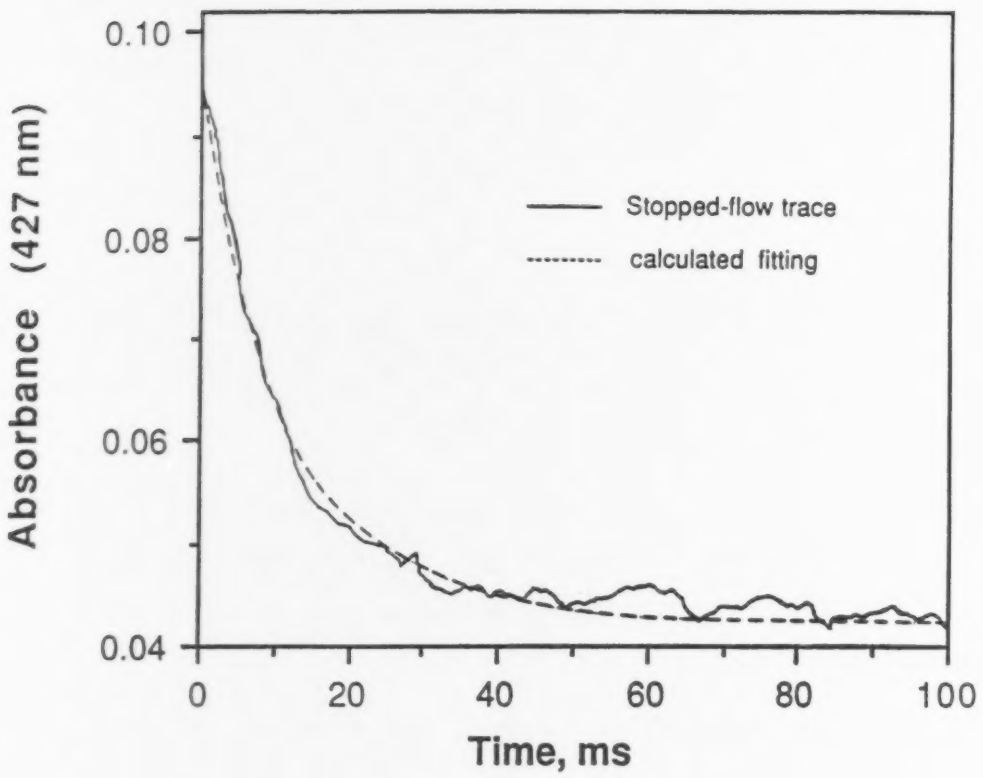


Figure 3.6. Stopped-flow trace of absorbance for the oxidation of 2-AF by HRP-II monitored at 427 nm. The reaction was carried out at pH 7.10 by means of a pH-jump. Ionic strength, 0.11 M; $[HRP\text{-}II]_0$, 1 μM; $[2\text{-}AF]_0$, 1 μM; temperature, 25°C.

corresponding HRP-I reactions, the reactions of HRP-II with 2-AF were also performed with the initial concentrations of the two reactants being equal. Stopped-flow traces of absorbance obtained at 427 nm show the time course of the disappearance of HRP-II. Fig. 3.6 shows such a hyperbolic trace and its fitting. The fitting yields the apparent second order rate constant, $k_{2,app}$.

The plot of log $k_{2,app}$ versus pH is shown in Fig. 3.7. The ionizations of two acid-base groups seem to play important roles in the reaction between HRP-II and 2-AF.

The rate constant for the reaction of 4-aminobiphenyl with HRP-II is (1.0 $\pm$ 0.3) x 10^7 M^{-1} s^{-1}.

3.5 DISCUSSION

Previous studies with HRP/H_2O_2/2-AF system showed that 2-AF can be activated to electrophilic intermediate(s) capable of binding to macromolecules (18). A one-electron oxidation mechanism involving free radicals was proposed, mainly based on metabolic product analysis (19). Though ESR spectroscopy failed to detect the signals from the proposed primary free radicals, the evidence for the one-electron oxidation may be inferred from the analysis of the metabolic products (19), or from the analysis

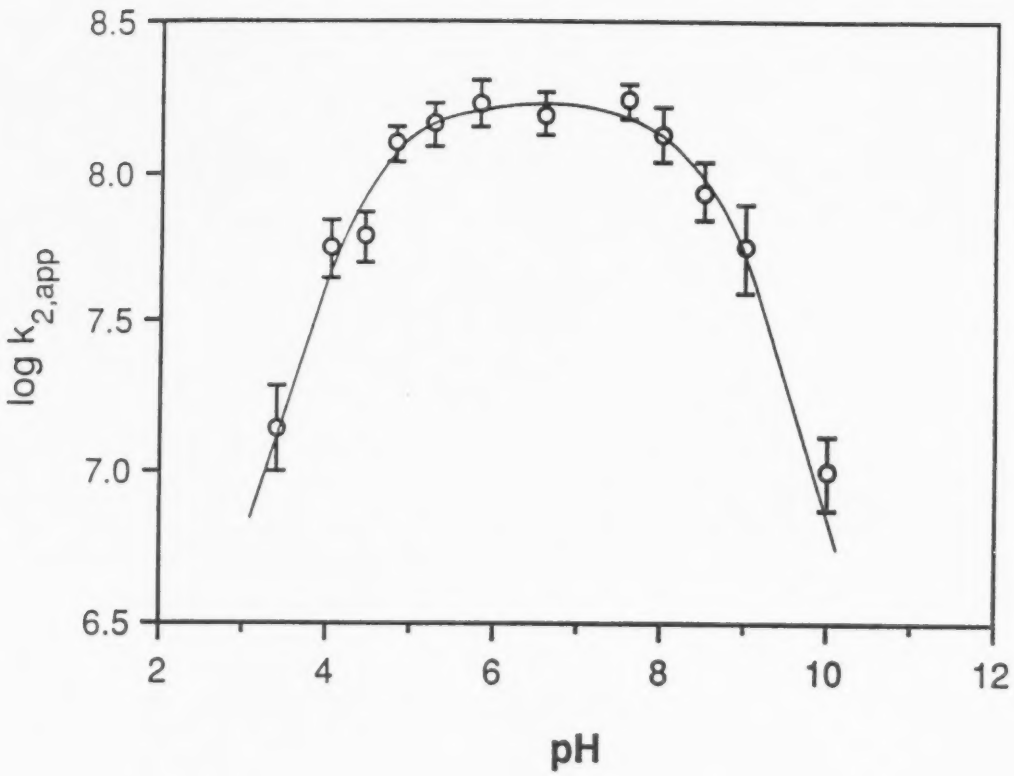


Figure 3.7. pH profile of second order rate constant for the reaction of HRP-II with 2-AF at 25°C and ionic strength 0.11 M. The curve was obtained through computation using eq. [3.6] on the basis of the best-fit parameters listed in Table 3.1.

of the enzyme oxidation state changes, which has been performed in this study.

The enzymatic cycle for HRP is dependent on the nature of the reducing substrate. Following the formation of the enzyme intermediate compound I, either two one-electron redox reactions or a two-electron redox reaction can complete an enzymatic cycle (23-28). Our experiments with HRP/H_2O_2/2-AF using rapid scan spectrophotometry indicate that HRP-I undergoes two serial one-electron reduction steps, and HRP-II undergoes a one-electron reduction step to native HRP, all of which can be described by the normal peroxidatic cycle (29,30):

$$\text{HRP} + H_2O_2 \longrightarrow \text{HRP-I} + H_2O$$

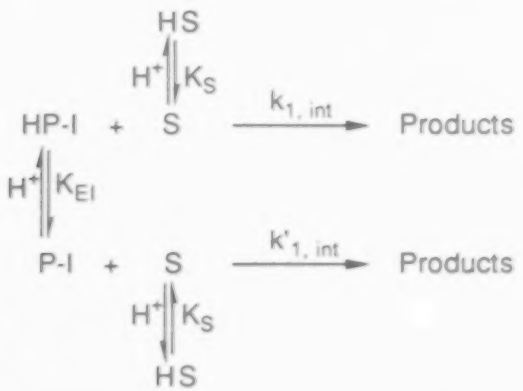
$$\text{HRP-I} + \text{R-NH}_2 \longrightarrow \text{HRP-II} + \text{R-NH}\bullet$$

$$\text{HRP-II} + \text{R-NH}_2 \longrightarrow \text{HRP} + \text{R-NH}\bullet + H_2O$$

where R denotes the 2-fluorenyl group. It is clear from this scheme that the free radical, R-NH•, is necessarily the primary product. Due to the further reactions of the free radical, its signal was not directly observed in our ESR experiments. However, work has been undertaken on the indirect ESR detection of the primary free radical product using spin trapping technique.

Based on the previous experiments showing that a distal group of pK_a~5.1 plays a role in HRP-I reactions (31), the following scheme is proposed to account for the pH profile of the reaction of HRP-I with 2-AF:

[3.3]



where $k_{1,\,int}$ and $k'_{1,\,int}$ represent the intrinsic rate constants of the two ionization states of HRP-I reacting with 2-AF, respectively. The above scheme leads to the following $k_{1,app}$ -pH relationship:

[3.4]

$$k_{1,app} = \frac{k_{1,\,int} + \dfrac{k'_{1,\,int}\,K_{EI}}{[H^+]}}{\left[1 + \dfrac{K_{EI}}{[H^+]}\right]\left[1 + \dfrac{[H^+]}{K_S}\right]}$$

The experimental data points are shown in Fig. 3.4. A non-linear least square analysis program was used to obtain the best-fit parameters which are listed in Table 3.1. The computed curve based upon the best-fit parameters can be

seen in Fig. 3.4. The agreement between the best fit pK_S and pK_{BH^+} of 2-AF obtained separately in this study, and the agreement between the best fit pK_E and the pK_a of a kinetically significant acid-base group of HRP-I comfirm that 2-AF is reactive when it is in its neutral form, and the deprotonation of a acid-base group of HRP-I slightly lowers its reactivity.

A different mechanism seems operative in the reaction between HRP-II and 2-AF:

[3.5]

$$\begin{array}{ccc} \text{HP-II} + \text{S} & \xrightarrow{k_{2,\,int}} & \text{Products} \\ H^+ \Big\| K_{EII} \quad H^+ \Big\| K_S & & \\ \text{P-II} + \text{HS} & & \end{array}$$

where $k_{2,int}$ is the intrinsic rate constant of HRP-II reacting with 2-AF. The corresponding $k_{2,app}$-pH relationship can be expressed as follows:

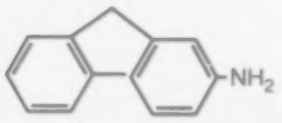
[3.6]

$$k_{2,app} = \frac{k_{2,\,int}}{\left[1 + \frac{K_{EII}}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_S}\right]}$$

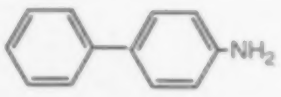
The fitting of the data points shown in Fig. 3.7 to eq. [3.6] gives the best fit values for pK_E and pK_S, which are listed in Table 3.1. The curve computed on the basis of these parameters is shown in Fig. 3.7. It can be seen again that

when it is protonated 2-AF becomes inactive toward oxidizing HRP. The deprotonation of a acid-base group at the active site of HRP-II removes its reactivity. The pK_a value of 8.6 for this group, observed in present study, is in agreement with the results of previous studies (31).

Our kinetic experiments show that 2-AF is a very effective reducing substrate for HRP compounds, as reflected from the intrinsic second order rate constants, $(1.1 \pm 0.5) \times 10^9\ M^{-1}s^{-1}$ for HP-I, $(4.8 \pm 0.4) \times 10^8\ M^{-1}s^{-1}$ for P-I , and $(1.7 \pm 0.2) \times 10^8\ M^{-1}s^{-1}$ for HP-II reactions. The Hammett $\rho\sigma$ correlations have been established and mechanistic information has been extracted therefrom for the oxidation of the substituted anilines by HRP-I and HRP-II (32,33). Though 2-AF (**I**) is not a simple substituted aniline, it is structurally close to 4-aminobiphenyl (**II**), a para-substituted aniline. Because of the similarity of **I** and **II**, it is instructive to examine the behavior of **II**. On the basis



(**I**)



(**II**)

of the Hammett $\rho\sigma$ relationship and the similar σ values for 4-aminobiphenyl and aniline, one would predict that 4-aminobiphenyl reacts with HRP-I and HRP-II at nearly the same rates as those of aniline, which at pH 7.00 and 25.0^0 C are $(2.4 \pm 0.2) \times 10^5\ M^{-1}s^{-1}$ and $(2.9 \pm 0.3) \times 10^4\ M^{-1}s^{-1}$, respectively

(32,33). The rate constants for 4-aminobiphenyl are larger by factors of 200 and 350 for HRP-I and HRP-II compared to the rate constants for aniline. It would appear that there is a change of mechanism. Therefore 4-aminobiphenyl does not react by transfer of a hydrogen atom (a proton plus an electron transferred simultaneously) as has been established for other substituted anilines and phenols (31-34). We propose that 4-aminobiphenyl reacts by electron transfer from its aromatic nucleus, therefore generating a π-cation radical. Since 2-AF reacts even faster, we also propose that it reacts in the same manner as 4-aminobiphenyl. The faster rate for 2-AF can be explained on the basis that it has a rigid structure, whereas some rotation can occur about the bond joining the two benzene rings of 4-aminobiphenyl, which would lower the aromaticity of the latter compound. The higher the degree of aromacity, the greater the stabilization of the π-cation radical (35). Thus 2-AF reacts even faster than 4-aminobiphenyl. Similar mechanisms were proposed for N,N-substituted aromatic amines and methoxybenzenes in their oxidations catalyzed by HRP, which also involve the formation of cation radical intermediates of the substrates (36,37).

The mechanism that 2-AF and 4-aminobiphenyl react with the oxidizing compounds of HRP through a simple electron jump is consistent with the published redox potentials for the enzyme system and the two substrates. For HRP-I / HRP-II and HRP-II / ferric HRP couples, redox potentials (E_0's) of ~ 950

mV were obtained from equilibria data coupled with the $K_2 I_r Cl_6$ - $K_3 I_r Cl_6$ system at slightly acidic pH values (38). Using hydrodynamic voltammetry, the redox potentials ($E_{1/2}$'s) for 2-AF and 4-aminobiphenyl were determined and the values of 530 mV and 850 mV were reported (39).

In conclusion, the rapid-scan spectral analysis of the conversions among enzyme intermediates confirms that HRP in the presence of H_2O_2 oxidizes 2-AF through a one-electron mechanism and a free radical must be generated as the primary product. The kinetic measurements and the pH dependence studies indicate that when it is in its neutral form 2-AF is a very effective reducing substrate for HRP-I and HRP-II in the presence of H_2O_2 . A mechanism involving a cation radical formed from the substrate in the rate determining step and stabilized by the extensive π-conjugation system of 2-AF may account for the extraordinarily high reaction rates.

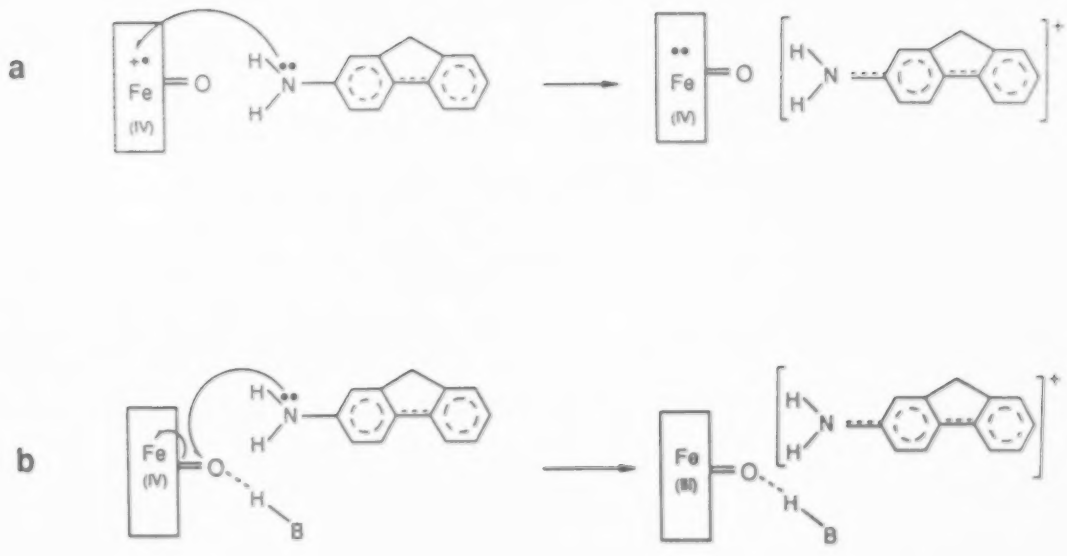


Figure 3.8. Hypothetic schemes showing the rate controlling step in the cases of HRP-I (a) and HRP-II (b) reacting with 2-AF. The rectangles represent the porphyrin rings. One electron transfers to the enzyme species yielding a cation radical in the substrate, which is then stabilized by the extensive π-conjugation system of 2-AF. B is a distal base hydrogen-bonded to the ferryl oxygen in HRP-II.

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CHAPTER FOUR

GENERAL DISCUSSION

Although the industrial usage of carcinogenic aromatic amines has been severely curtailed nowadays, significant human exposure to this type of carcinogen still occurs. Cigarette smoke, for example, contains 2-naphthylamine and 4-aminobiphenyl (1), and these may be responsible for the positive correlation between cigarette smoke and bladder cancer in humans (2). In addition, carcinogenic heterocyclic aromatic amines formed by the pyrolysis of amino acids during the preparation of certain foods may be important in the etiology of human cancer (3). There also appears to be widespread exposure to nitro-PAHs (nitro-polycyclic aromatic hydrocarbons), which result from the incomplete combustion of organic material such as diesel fuels and are converted to aromatic amines by nitro-reduction (4). The incidence of human exposure to aromatic amines seems to be extensive as indicated by the almost universal formation of 4-aminobiphenyl hemoglobin adducts in human blood (5). The source of this contamination is unknown at present. The chemical carcinogenesis of aromatic amines will continue to be an active research area.

The structure-activity relationship for the carcinogenicity of aromatic amines has not been clearly established. It is expected that some information concerning this relationship can be obtained through investigation of their metabolic activation. In order to study the reactivity of aromatic amines in peroxidase-mediated oxidative metabolism, two types of aromatic amines were chosen as the substrates for the HRP/H_2O_2 oxidizing enzyme system: (i) a series of monosubstituted anilines, and (ii) two model carcinogenic arylamines, 2-aminofluorene and 4-aminobiphenyl. Experimental results have shown significantly different reactivities, suggesting different reaction mechanisms for the two types of aromatic amine substrates. The reactivity of monocyclic substrates depends upon the substitution on the ring, which is in accordance with the Hammett relationship. However, the two polycyclic substrates show extraordinarily high reactivities, which clearly do not conform to the Hammett equation. Two mechanisms were proposed to account for the discrepancy. While the monocyclic aromatic amines are believed to undergo the oxidation through a hydrogen atom transfer forming a nitrogen-centered free radical, the oxidation of the polycyclic arylamines is proposed to proceed through an electron transfer, generating a cation radical which is effectively stabilized by the extensive π-conjugation system of the polycyclic aromatic molecules.

It has been known that not all aromatic amines are potent carcinogens. Empirical data suggest that the nature of the substitution in the molecule and the type of aromatic ring system are important structural factors (6). The simplest aromatic amine, aniline, is considered a non-carcinogen. Methoxyl substitution on the the ring, such as p-anisidine, yields a weak carcinogen (7). The most active amine carcinogens contain two or more aromatic rings. These include 2-naphthylamine, 4-aminobiphenyl, 2-aminofluorene, benzidine, and also compounds in which two benzene rings are separated by an azo bond such as 4-aminoazobenzene (Fig. 1.3).

As can be seen from the experimental results in this study, the two major structural factors governing the carcinogenic activity of aromatic amines, i.e., the nature of the substitution in the molecule and the type of aromatic ring system, are also the major structural features dictating the reactivity of aromatic amines toward peroxidase mediated oxidative metabolism. This fact leads to a speculation that the faster formation and the greater stability of the radical product of oxidation may yield the stronger carcinogenicity of an aromatic amine. This speculation is also supported by the following facts:

(i) Generally there are different metabolizing pathways which co-exist in human tissues and compete for xenobiotics such as a potent carcinogen. Whereas some of the pathways can activate the potent carcinogen to an ultimate carcinogenic species, others may represent detoxification

mechanisms. The relative importance of individual pathways to the overall carcinogenic activity should be dependent on the relative reactivity of the chemical towards an individual metabolizing system. Although a numerical reactivity-carcinogenicity correlation is impossible currently because of the lack of a quantitative description of carcinogenicity, a qualitative correspondence does exist for the substances, aniline, p-anisidine, and 2-aminofluorene. Thus both the reactivity toward peroxidase catalyzed oxidation and the observed carcinogenicity follow the same order: aniline < p-anisidine < 2-aminofluorene.

(ii) The relative degree of DNA binding of arylamines oxidized by peroxidases was found to be dependent on the stability of each radical product (8). The suggestion can be made that a radical species has to be sufficiently long-lived to penetrate the nuclear membrane and react with DNA bases. In other words, DNA binding cannot occur unless the radical metabolite can reach DNA bases before the annihilation through other reactions, such as polymerization. It can be inferred that aromaticity, the structure-derived property, is one of the determinant factors for the carcinogenic potential of aromatic amines since aromaticity and free radical stability can be correlated. Thus an explanation can be found why high degree of carcinogenic activity has been detected in polycyclic aromatic amines and why monocyclic aromatic amines are generally lower in

carcinogenic potential, even though some of them have shown reactivities as high as those of polycyclic species in peroxidase catalyzed oxidation (9,10).

There has been an increased interest in the study of peroxidase involved metabolic activation of chemical carcinogens in recent years. Although HRP is considered as an ideal model for mammalian peroxidases in such studies, exceptions do exist when such aspect as product profiles of relevant reactions are concerned. More peroxidases from mammalian sources should be employed in the future studies to better mimic *in vivo* oxidative metabolism of carcinogenic aromatic amines and other chemical carcinogens.

As the result of continuing effort of scientists, a vast amount of data on the carcinogenic activity of aromatic amines has been accumulated. In contrast, reactivity data for aromatic amines in their reactions with metabolizing enzyme systems are still quite limited. Considerable more work is required to improve this situation. The carcinogenicity data collected in more than one hundred years is a "treasure-house", which, if combined with corresponding data for the reactivity of the carcinogens toward metabolizing enzyme systems, would greatly facilitate the establishment of the structure-activity relationship of chemical carcinogens and enable us to carry our understanding of chemical carcinogenesis to a new level.

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PART II

MYELOPEROXIDASE REACTIONS WITH HYDROGEN PEROXIDE

CHAPTER FIVE

INTRODUCTION

Myeloperoxidase (MPO) was first isolated and characterized by Agner in 1941(1,2). Starting with empyemic fluid from tuberculosis patients, or leukocytes from patients with myeloid leukemia, a hemoprotein was obtained which was initially termed verdoperoxidase, a name derived from its green color. This enzyme is now well known, mainly for its role in phagocytosis, a process which constitutes an important cellular defence mechanism against invading pathogens (3-5).

5.1 PHAGOCYTOSIS: ROLE OF MYELOPEROXIDASE

In the late 1800's, the Russian zoologist Elie Metchnikoff observed the engulfment of bacteria by cells from the bloodstream of animals (6). This process is called phagocytosis -- the cell "flows around" the foreign particle and encloses it in a plasma membrane vesicle that becomes internalized into the cytoplasm of the phagocytic cell. Phagocytic cells (or phagocytes) in the

human bloodstream are neutrophils which have a multilobed nucleus (hence they are called polymorphonuclear cells, or PMNs) and a large number of cytoplasmic granules. The primary or azurophile granules contain the enzymes myeloperoxidase and lysozyme, several proteases, and a number of granular cationic proteins.

The phagocyte response to microbial invasion includes several phases: (i) migration to the site of injury; (ii) ingestion; (iii) degranulation; and (iv) death of the microbe. The first phase is initiated by the release of attracting substances (chemoattractants) from the inflammatory site. Under the influence of these chemical messages, the white blood cells (WBC) leave the circulating blood (7) and adhere to the capillary walls (8). They then move through the walls into the tissue spaces and arrive at the site of injury. The second phase begins when microbe and phagocyte make physical contact. Usually plasma proteins, immunoglobulins (IgG and IgM) and complement factors (C_{3b}), assist in this step (9). With the contact firmly established, projections of WBC cytoplasm (pseudopodia) flow about the microorganism, enclosing it in a vacuole referred to as a phagosome. The cytoskeletal apparatus of the phagocyte, particularly the microfilament system, facilitates this enclosure (10). Even as the ingestion is occurring, the third phase, degranulation, is initiated as cytoplasmic granules migrate toward the phagosome; they fuse with it and discharge their contents, including MPO, directly into the space

around the microbe (11). The interaction of these granule constituents with reactive oxygen species elaborated by the phagocytes leads ultimately to the fourth and final phase: death of microorganisms (12).

The reactive oxygen species involved in phagocytosis are generated in a process termed the "respiratory burst" (13). When stimulated, the neutrophils cause a burst of oxygen consumption in which a membrane-associated flavoenzyme utilizes NAD(P)H to convert molecular oxygen to superoxide (14,15):

$$2O_2 + NAD(P)H \xrightarrow{\text{flavoenzyme}} 2O_2^{\cdot} + NAD(P)^+ + H^+$$

Superoxide dismutes, either spontaneously or catalyzed by superoxide dismutase (SOD), to form hydrogen peroxide:

$$2O_2^{\cdot} + 2H^+ \xrightarrow{\text{(SOD)}} H_2O_2 + O_2$$

It is believed that the real killing of the microorganism involves the MPO-H_2O_2-X^- (halide) system (16-18). In the presence of hydrogen peroxide, and chloride or iodide ions, MPO can kill a number of bacteria and fungi *in vitro* . Since there are more chloride than iodide ions in the phagocyte cytoplasm, it has been suggested that MPO carries out the killing by oxidizing

Cl^- to hypochlorous acid, HOCl. Being a very strong oxidizing agent, hypochlorous acid can oxidize many biological molecules, thus it can kill bacteria. Common household bleach contains HOCl; it is used to disinfect hospital operating rooms.

MPO is also implicated as a modulator of the inflammatory response. Hypochlorous acid produced by MPO-H_2O_2-Cl^- system activates collagenase which is involved in the degradation of collagen. The destruction of collagen plays a key role in the development of inflammatory disease states affecting every organ system in the human body (19). Therefore, there are both positive and negative biological consequences that derive from the reaction products of the MPO system (20).

5.2 STRUCTURE AND PROPERTIES OF NATIVE MPO

The important physiological functions of MPO has stimulated great interest in the investigation of the structure and properties of the enzyme. The uniqueness of the enzyme is now widely acknowledged; it is due to the complexity of its structure and the difference of its prosthetic group from that of other peroxidases.

MPO has a relatively complex subunit structure. While most studies agree that the enzyme is a heterodimer consisting of two heavy-light protomers (21-24), there have been discrepancies regarding the molecular

mass of the enzyme as well as those of the subunits. In addition, the interdependence and the equivalence between the two heavy-light protomers are also of controversy. The value of molecular mass obtained for MPO of different sources and by different methods ranges from 130 to 153 kDa (21-30). The native enzyme has been successfully cleaved into "hemi-MPO" by reductive alkylation, each half consisting of a heavy and light subunit with the heavy unit being ~ 57.5 kDa and the light unit ~14 kDa. The hemi-MPO maintains the same specific activity as the native complete enzyme, suggesting the equivalence and excluding an interdependence between the two halves of the enzyme (31,32).

MPO has two heme groups, each covalently linked to a heavy subunit (27,33,34). The nature of the heme in MPO has been controversial. Optical properties of MPO (35), including magnetic circular dichroic spectra (36), are consistent with a chlorin-type heme (Fig. 5.1). The optical data, however, cannot exclude the alternative possibility that it is a heme possessing strongly electron-withdrawing substituent groups. These would red-shift the visible and Soret bands from their usual spectral positions, e.g., as occurs in formyl-containing A-type porphyrins (37). Recent studies using resonance Raman (RR) spectroscopy have confirmed that the prosthetic group in the intact enzyme is a chlorin, the distinguishing features being the large number of

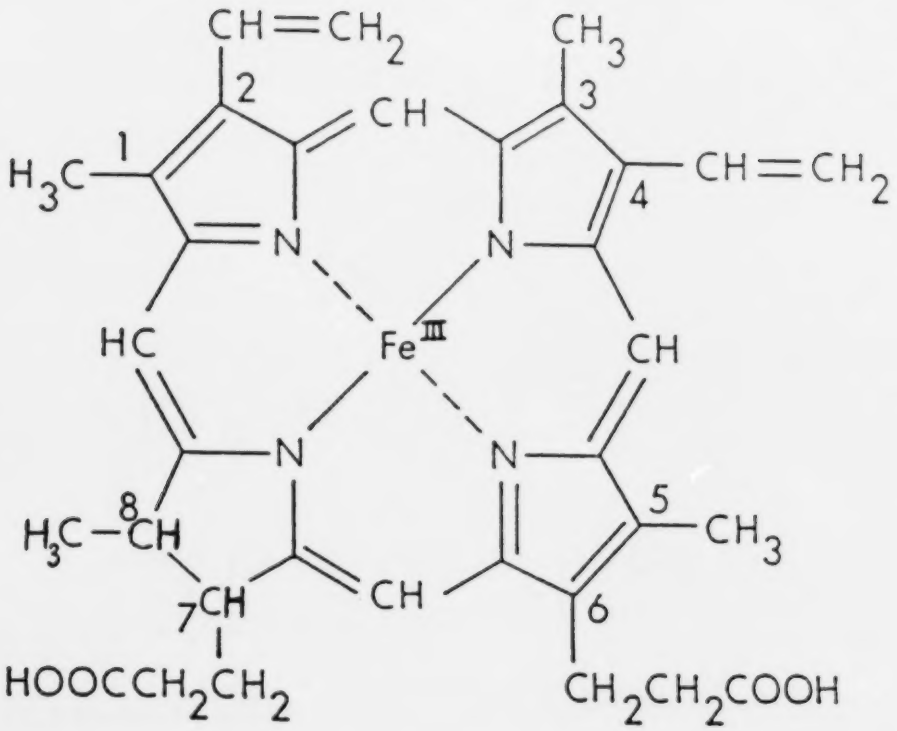

Figure 5.1. Structure of a chlorin-type heme: proposed prosthetic group of myeloperoxidase. Compared to ferriproyoporphyrin IX (Fig. 1.1), conjugation in of the the pyrrole rings is disrupted by reduction across the β-carbon double bond.

bands associated with coupled vibrational modes of the macrocyclic ring (a consequence of the lower symmetry of chlorins vs. porphyrins), and the absence of any bands attributable to formyl or other electron-withdrawing substituents conjugated to the ring (38-41).

The heme in MPO is more tightly bound to the apoenzyme compared to those in other peroxidases. Upon the addition of acidified acetone, for example, the release of the heme and the precipitation of the glycoprotein are observed for these peroxidases whereas for MPO the whole molecule precipitates without the cleavage of prosthetic group from glycoprotein (42). The enzyme has the ability to retain 100% of its activity at extreme pH values (2.45 and 12.8), even at 60^0C (43). The stability of the enzyme may partly be attributed to the disulfide bridges in the molecules. It has been shown that a very high cysteine content exists in MPO (44).

The gene sequence for MPO in its precursor polypeptide form has recently been determined, from which the amino acid sequence has been deduced (45,46). The residue at 416 has been proposed as possible candidate for the proximal histidine residue that is bound to the iron center of the heme. While the existence of a distal His and a distal Arg has been indicated as an important feature of other peroxidases, the corresponding locations in MPO are still under investigation.

5.3 ENZYME INTERMEDIATES AND THEIR REACTIONS

MPO, like other peroxidases, catalyzes the H_2O_2-dependent oxidation of many organic compounds including phenols and aromatic amines. However, the physiological functions of MPO and other animal peroxidases are mainly derived from their capacity of peroxidizing halide and pseudohalide ions.

As the first step in the catalytic mechanism of peroxidases, the enzymes react with peroxides. Hydrogen peroxide is probably the physiological substrate, though the enzymes also react with related compounds such as alkyl hydroperoxides (ROOH). The reaction with peroxides converts the enzyme from its native form to the compound I state, which contains two oxidizing equivalents above that of the native state in its structure. Compound I of peroxidases oxidizes halide ions and returns to its native state in a single two-electron transfer reaction (Fig. 5.2).

The halide specificity is different among peroxidases. No peroxidase catalyzes F^- oxidation, but MPO catalyzes oxidation of all other halide ions (47). Eosinophil peroxidase (EPO) is much less active in Cl^- oxidation, but is highly active with Br^-, SCN^-, and I^- (48-50). Lactoperoxidase (LPO) has barely detectable activity with Br^-, but catalyzes the oxidation of SCN^- and I^- (47). Thyroid peroxidase (TPO) may have the same specificity as LPO (51), whereas horseradish peroxidase (HRP) is active only with I^- (47). The halide

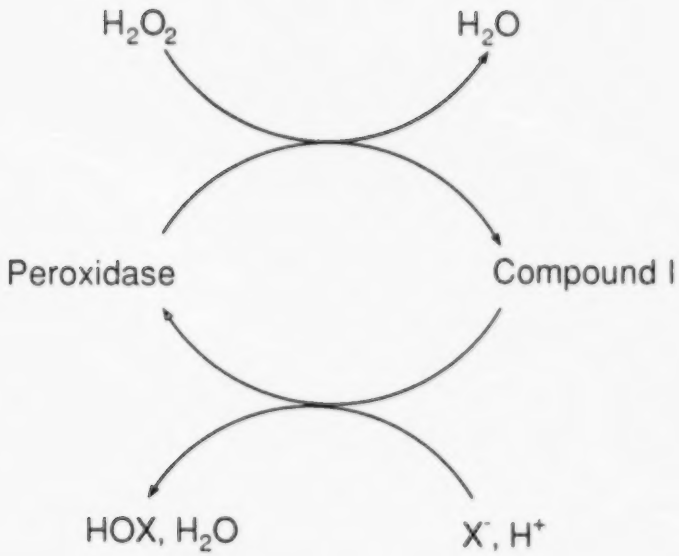

Figure 5.2. The catalytic cycle of peroxidase in halide oxidation.

specificity of the peroxidases suggests that the compound I states form a series with decreasing oxidation potential: MPO > EPO > LPO ~ TPO > HRP.

The high oxidation potential of MPO compound I (MPO-I) may contribute to its instability. It is known that MPO-I can undergo one-electron spontaneous reduction to compound II (MPO-II), the mechanism of which has not been established. Related to this decay is the finding that it is difficult to convert all of the native enzyme to compound I by adding hydrogen peroxide, even in the absence of electron of donors. MPO-II, or a mixture of MPO-I and MPO-II, is usually obtained.

As with other peroxidases, MPO-I is reduced sequentially to the native enzyme via MPO-II by a wide variety of organic compounds capable of mediating one-electron oxidation. Because MPO-II does not oxidize chloride, molecules which act as one-electron donors to MPO-I inhibit HOCl formation by the MPO-H_2O_2-Cl^- system. This type of inhibition is transient because electron donors eventually reduce compound II to the native state. However, the transient inhibition can be significant when the enzyme is competing for hydrogen peroxide with other enzymes such as catalase (51-53).

Reaction of superoxide with MPO in neutrophils or the extracellular medium converts the enzyme to another oxidation state, compound III (MPO-III), which contains a complex, [Fe(III) --- O_2^- <----> Fe(II) --- O_2] (54,55). MPO-III is inactive in Cl^- oxidation but can be converted to active forms. It has been

suggested that superoxide can convert MPO-III to MPO-I (56). Hydrogen peroxide, on the other hand, has been shown to convert MPO-III to MPO-II (57).

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CHAPTER SIX

A NEW MECHANISM FOR MPO REACTIONS WITH HYDROGEN PEROXIDE*

6.1 SUMMARY

The conversion of myeloperoxidase to its two oxidized intermediates, compounds I and II, in the presence of hydrogen peroxide has been investigated. Rapid scan studies indicate that at least a 100-fold excess of hydrogen peroxide is required to obtain a spectrum of pure compound I; the stability of compound I so obtained decreases as the concentration of hydrogen peroxide increases. The time courses for both the decay of compound I and the formation of compound II are biphasic. Either increasing hydrogen peroxide concentration or adding superoxide dismutase to the system results in the convergence of the two time courses to monophasic processes. A new mechanism has been developed to account for the experimental results reported herein. According to this mechanism, compound I is formed in a reversible reaction, but not as a simple Michaelis-

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* Abbrieviations: MPO, myeloperoxidase (EC 1.11.1.7 donor-H_2O_2 oxidoreductase) ; MPO-I, myeloperoxidase compound I; MPO-II, myeloperoxidase compound II; SOD, superoxide dismutase.

Menten type complex. Compound I further reacts with hydrogen peroxide to form compound II and superoxide. Superoxide, once formed, also reduces compound I to compound II with the concomitant generation of oxygen. The roles of hydrogen peroxide and superoxide as the reducing substrates in the conversion of compound I to compound II explain the abnormal stoichiometry for the reaction of myeloperoxidase with hydrogen peroxide and the spontaneous decay of compound I.

6.2 INTRODUCTION

Myeloperoxidase is an enzyme found in mammalian neutrophils and monocytes and involved in the host defense against invading microorganisms (1). Although its chemical structure has not yet been fully elucidated, the enzyme is known to have two hemes as its prosthetic groups and contain four subunits ($\alpha_2\beta_2$) in its protein moiety (2) .

Myeloperoxidase is one member of the peroxidase family. As two-substrate enzymes, peroxidases undergo the following peroxidatic cycle in the presence of a hydroperoxide, ROOH, and an electron donor, AH_2 (3,4):

native enzyme + ROOH $\longrightarrow$ compound I + ROH

compound I + AH_2 $\longrightarrow$ compound II + •AH

compound II + AH_2 $\longrightarrow$ native enzyme + •AH + H_2O

However, the *in vivo* peroxidation of chloride ion catalyzed by myeloperoxidase differs from the above mechanism:

MPO-I + Cl^- $\longrightarrow$ MPO + OCl^-

where the only oxidized intermediate involved is MPO-I, which is directly reduced to native MPO (5). The product of the reaction, hypochlorous acid, is responsible for much of the antimicrobial activity of the MPO/H_2O_2/Cl^- system (6,7).

Previous studies have revealed unique behavior of myeloperoxidase in the presence of hydrogen peroxide. Horseradish peroxidase, taken as an example of many other peroxidases, forms relatively stable compound I with a half-life of 15-20 min upon the addition of equimolar hydrogen peroxide (8). Compound I of myeloperoxidase, however, is very unstable, and spontaneously decays to its one electron reduction product, compound II (9). Also different is the stoichiometry for the formation of compound I and compound II; a large excess of hydrogen peroxide is required for myeloperoxidase (9,10).

Considerable effort has been made to explore the mechanism behind the unique behavior of myeloperoxidase in its reaction with hydrogen peroxide. Among the proposed mechanisms are a Michaelis-Menten type complex formed as compound I (11), a catalase activity of myeloperoxidase (12), an intramolecular redox reaction between the two hemes of the enzyme (12), and a reaction of compound I with hydrogen peroxide (13,14).

In the present study we report our investigation of the formation and the spontaneous decay of myeloperoxidase compound I in the presence of

different concentrations of hydrogen peroxide. Superoxide dismutase is also employed to probe the involvement of superoxide in the reactions. A new mechanism is proposed for the formation of MPO-I and its conversion to MPO-II, which can explain some puzzling phenomena observed by different research groups.

6.3 MATERIALS AND METHODS

Bovine spleen myeloperoxidase was isolated and purified using a combination of published procedures with minor modifications (15-17). Macerated beef spleens were suspended in 0.25 M KCl at 4^0C, and then homogenized in a Waring blender at maximum speed for 2 minutes. The pH of the homogenate was adjusted to 3.5 with 6 M HCl. The acidic homogenate was stirred at room temperature for 5 hours then centrifuged at 10,400 x g in a Sorvall-type GSA rotor with a Sorvall RC2-B centrifuge for 15 minutes. The supernatant was filtered through glass wool to remove small waxy particles. Citric acid was added to the filtrate to a final concentration of 0.1 M, followed by the pH adjustment to 5.5 with 12% NaOH. The solution was then treated with pre-cycled Whatman P11 cellulose phosphate fibrous cation exchanger and stirred for 1 hour. The cation exchanger was washed with water then resuspended with stirring in 2.0 M KCl for 2 hours followed by a filtration. The

filtrate was centrifuged at 10,000 x g for 20 minutes. The supernatant was concentrated using an Amicon ultrafiltration apparatus and then diluted to an ionic strength of 0.1 M, which is measured using a Yellow Spring Instrument Model 31 conductivity bridge and a KCl standard curve. The crude enzyme solution was then loaded to a CM-Sepharose CL-6B column and eluted using a linear ionic strength-pH gradient apparatus with 0.1 M phosphate buffer pH 6.0 as the starting buffer and 1.0 M phosphate buffer pH 7.0 as the limiting buffer. The further purification was done through gel permeation chromatography using a Sephacryl -200 column. Fractions with RZ values ($A_{430\ nm}$ / $A_{280\ nm}$) of 0.80 or higher were collected and dialyzed against deionized water before use. The concentration of the enzyme solution was determined spectrophotometrically using an extinction coefficient at 430 nm of 178 mM^{-1} cm^{-1} per molecule of enzyme (18).

Cu/Zn SOD from bovine erythrocytes and MnSOD from *E. coli* were purchased from Sigma. The concentration of the dissolved enzyme was determined spectrophotometrically using an absorbance coefficient of 15.9 mM^{-1} cm^{-1} at 265 nm for Cu/Zn SOD (19) and 36.8 mM^{-1} cm^{-1} at 282 nm for MnSOD(20).

Hydrogen peroxide was obtained from BDH Chemicals as a 30% solution, and the concentration of the diluted solution was determined using the horseradish peroxidase assay (21). Buffers were of reagent grade and

used without further purification. Solutions were prepared using deionized water from the Milli-Q system (Millipore).

Rapid scan and stopped-flow experiments were performed using a Photal Rapid Reaction Analyzer (Model RA-601) equipped with a 1 cm observation cell thermostated at 25.0 ± 0.5 ^{0}C. The rapid scan absorption spectra were measured by means of a multichannel photodiode array and stored in a computer interfaced to the Rapid Reaction Analyzer. A spectral region of 96 nm was scanned centered at about 440 nm. Spectral changes were recorded after 0.8 μM MPO in 0.1 M pH 7.0 phosphate buffer was mixed with H_2O_2 of different concentrations in the same buffer.

The time courses of the conversion of MPO-I to MPO-II were followed by monitoring both the decay of MPO-I and the formation of MPO-II in the stopped-flow experiments. One reservoir contained 0.8 μM MPO in 0.1 M pH 7.0 phosphate buffer; the other contained H_2O_2 of different concentrations (20-250 fold excess of the enzyme concentration) in the same buffer. The decay of MPO-I was observed at 442 nm, the isosbestic point between MPO and MPO-II, and the formation of MPO-II was observed at 456 nm, the isosbestic point between MPO and MPO-I.

Another set of experiments was also conducted under the same conditions, except that SOD was added. Both Cu/Zn SOD from bovine erythrocytes and MnSOD from *E. coli* used in this study.

6.4 RESULTS

Rapid scan spectra showing the formation and the decay of MPO-I

Different initial $[H_2O_2]/[MPO]$ ratios have been used to observe the formation and the decay of MPO-I. Small excesses of hydrogen peroxide give rise to incomplete formation of compound I before it starts to decay (Fig. 6.1). As the ratio $[H_2O_2]/[MPO]$ increases more complete formation of compound I is observed, but also the decay of compound I to compound II becomes faster (Fig. 6.2).

Stopped-flow traces showing the conversion of MPO-I to MPO-II

The conversion of MPO-I to MPO-II was followed through both the disappearance of MPO-I and the appearance of MPO-II. The corresponding time courses are shown in Figs. 6.3 and 6.4. It can be seen that the traces are similar for the decay of MPO-I and the formation of MPO-II. Two features of these time courses are worthy of note: (1) they are biphasic, and (2) they are dependent upon H_2O_2 concentration.

Effects of SOD on the conversion of MPO-I to MPO-II

Whereas a small amount of SOD has little effect on the time courses of the conversion of MPO-I to MPO-II, the conversion becomes faster (Fig. 6.5)

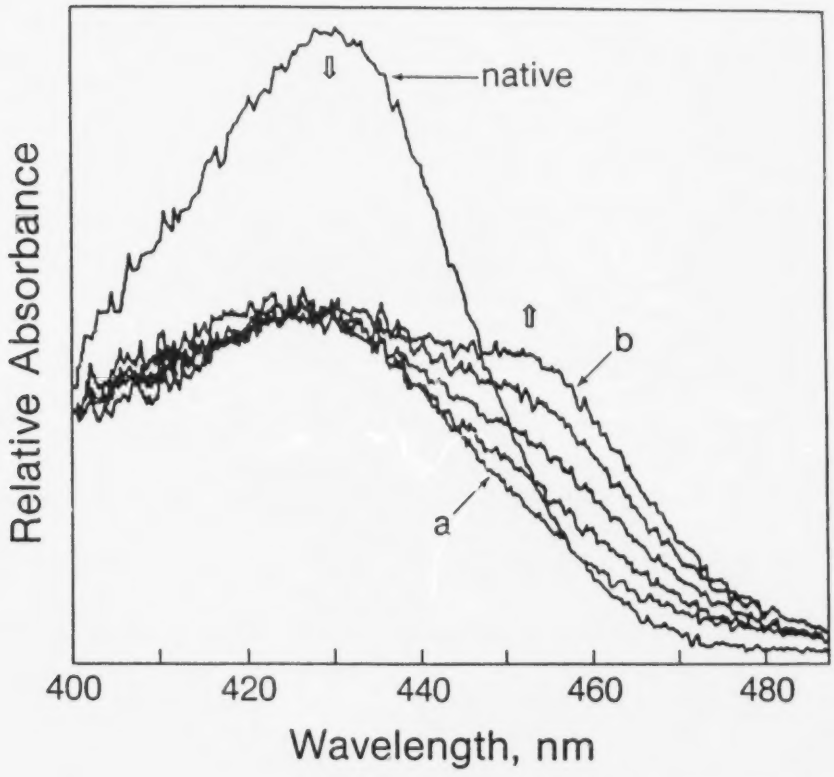


Figure 6.1. Rapid scan spectra showing the incomplete formation of compound I of myeloperoxidase by using a 30-fold excess of hydrogen peroxide. Final concentrations: 0.4 μM enzyme, 12 μM H_2O_2 in 0.1 M pH 7.0 phosphate buffer at 25^0 C. Spectum *a* was recorded at 1 ms and *b* at 166 ms after mixing. Arrows indicate direction of absorbance changes with time.

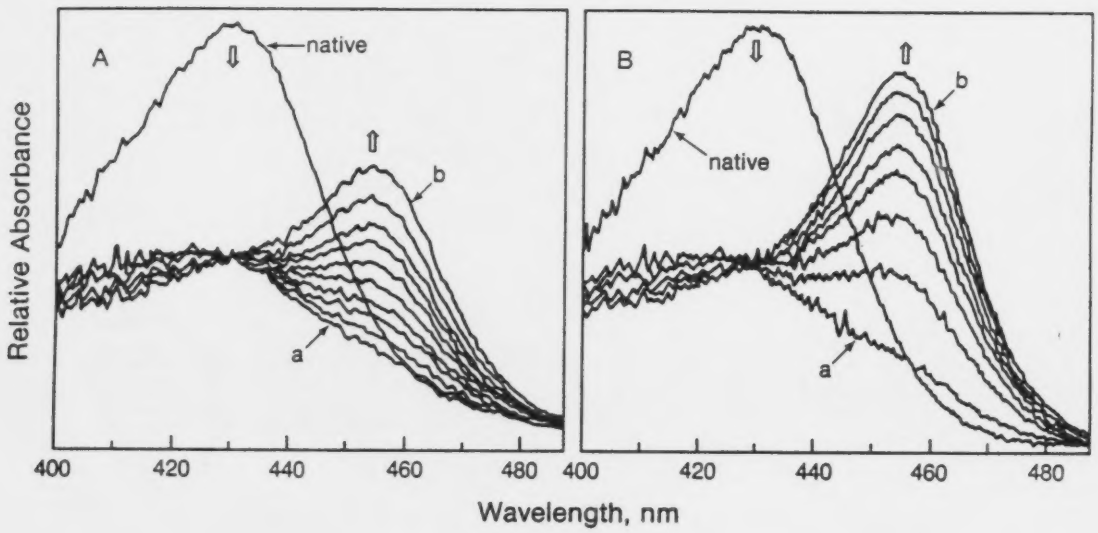


Figure 6.2. Rapid scan spectra showing the more complete formation and the faster decomposition of compound I by using larger excesses of hydrogen peroxide than in Fig.6.1. Final concentrations: (A) 0.4 μM enzyme and 40 μM H_2O_2 ; (B) 0.4 μM enzyme and 100 μM H_2O_2. Reactions were carried out in 0.1 M pH 7.0 phosphate buffer at 25^0 C. Spectrum *a* was recorded at 1 ms and *b* at 166 ms after mixing. Arrows indicate direction of absorbance changes with time.

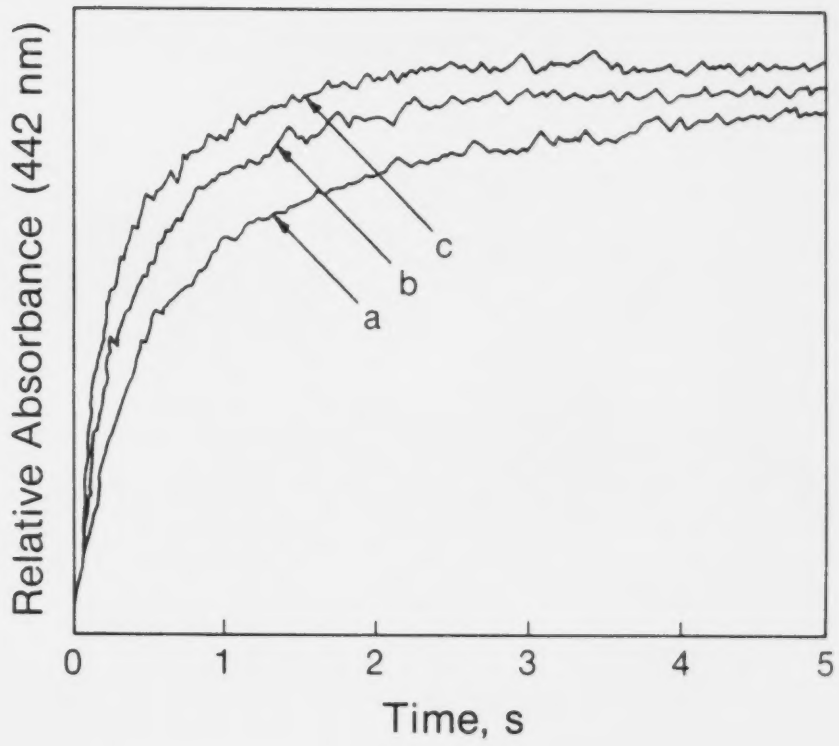


Figure 6.3. Time course traces of compound I decay at (a) 20-fold excess H_2O_2 , (b) 50-fold excess H_2O_2 , and (c) 100-fold excess H_2O_2 . Absorbance changes were followed at 442 nm, the isosbestic point between MPO and MPO-II. Final concentration of enzyme: 0.4 μM in 0.1 M pH 7.0 phosphate buffer.

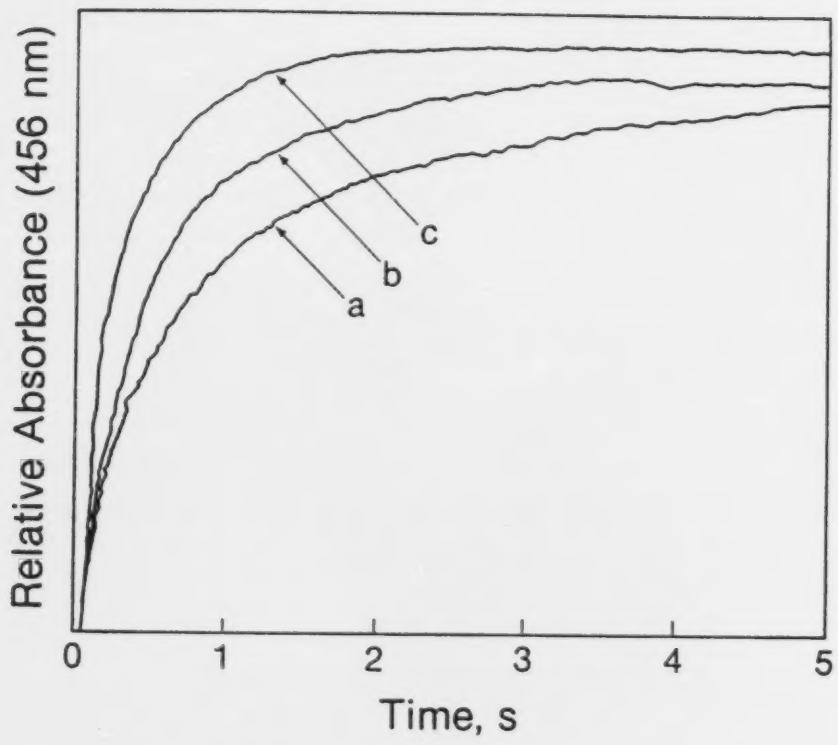


Figure 6.4. Time course traces of compound II formation at (a) 20-fold excess H_2O_2 , (b) 50-fold excess H_2O_2 , and (c) 100-fold excess H_2O_2 . Absorbance changes were followed at 456 nm, the isosbestic point between MPO and MPO-I. Final concentration of enzyme : 0.4 μM in 0.1 M pH 7.0 phosphate buffer.

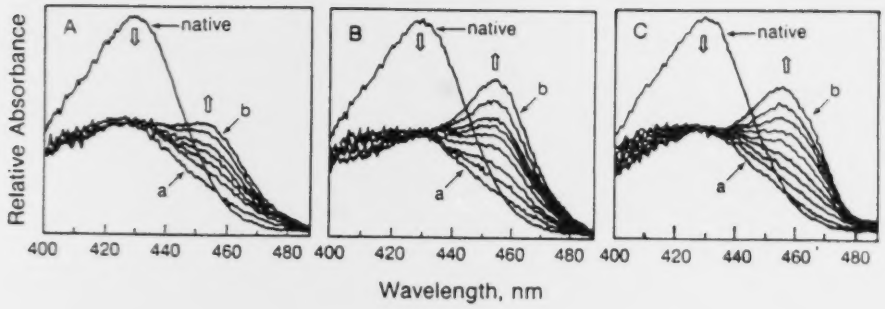


Figure 6.5. Effect of SOD on the conversion of MPO-I to MPO-II as reflected in the rapid spectral changes. Spectra were recorded under the following conditions: (A) One reservoir contained 0.8 μM myeloperoxidase, and the other, 48 μM hydrogen peroxide. (B) One reservoir contained 0.8 μM myeloperoxidase and 10 μM Cu/Zn SOD while the other contained 48 μM hydrogen peroxide. (C) One reservoir contained 0.8 μM myeloperoxidase and 10 μM MnSOD while the other contained 48 μM hydrogen peroxide. All reactions were carried out in 0.1 M pH 7.0 phosphate buffer at 25 ^{0}C. Spectrum *a* was recorded at 1 ms and *b* at 166 ms after mixing. Arrows indicate direction of absorbance changes with time.

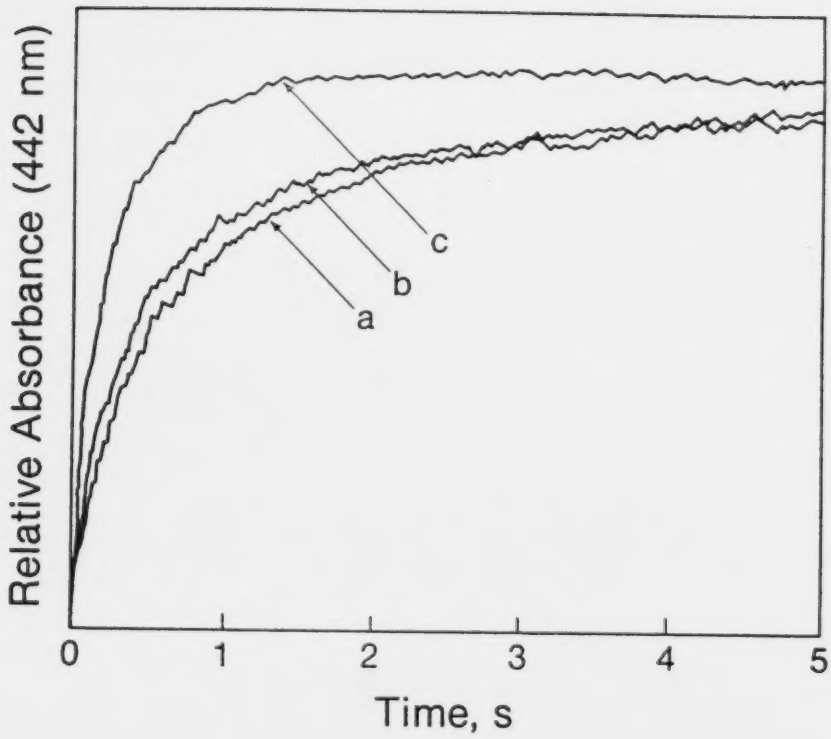


Figure 6.6. Effect of Cu/Zn SOD of different concentrations on the conversion of MPO-I to MPO-II as reflected in the time courses of MPO-I decay. Absorbance changes were followed at 442 nm, the isosbestic point between MPO and MPO-II. One reservoir contained 16 μM hydrogen peroxide. The other contained 0.8 μM myeloperoxidase (a), the mixture of 0.8 μM myeloperoxidase and 1 μM Cu/Zn SOD (b), or the mixture of 0.8 μM myeloperoxidase and 10 μM Cu/Zn SOD (c). All reactions were carried out in 0.1 M pH 7.0 phosphate buffer at 25 ^{0}C.

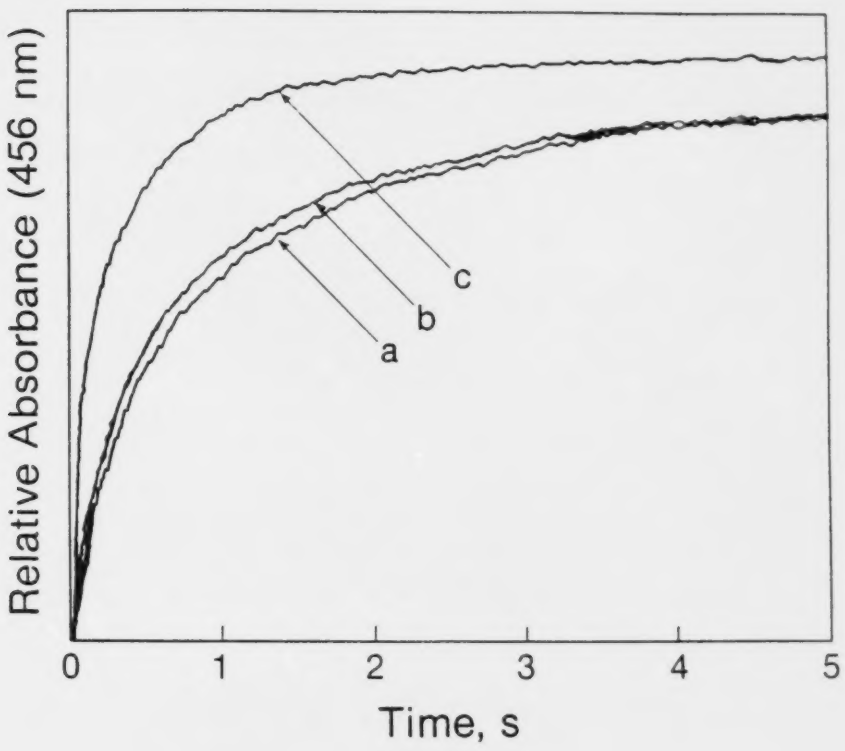


Figure 6.7. Effect of MnSOD of different concentrations on the conversion of MPO-I to MPO-II as reflected in the time courses of MPO-II formation. Absorbance changes were followed at 456 nm, the isosbestic point between MPO and MPO-I. One reservoir contained 16 μM hydrogen peroxide. The other contained 0.8 μM myeloperoxidase (a), the mixture of 0.8 μM myeloperoxidase and 1 μM MnSOD (b), or the mixture of 0.8 μM myeloperoxidase and 10 μM MnSOD (c). All reactions were carried out in 0.1 M pH 7.0 phosphate buffer at 25 ^{0}C.

and monophasic (Figs. 6.6 and 6.7) when either Cu/Zn SOD or MnSOD is added in higher concentrations.

6.5 DISCUSSION

Myeloperoxidase is a tetrameric protein which contains two hemes per molecule. In contrast to other peroxidases, myeloperoxidase has a prosthetic group with properties of an iron chlorin (22, 23) but also similar to the heme of lactoperoxidase, covalently linked through a sulfur atom (24). Thus myeloperoxidase is different from other peroxidases, which usually are monomeric and contain ferriprotoporphyrin IX as the prosthetic group (25).

Of the oxidized intermediates of myeloperoxidase, compound I is the only species which takes part in both the peroxidatic cycle and the chlorinating reaction (26). Despite its significant roles in these reactions, some mechanistic details about compound I remain unsolved. Among the puzzles are the abnormal stoichiometry for compound I formation and the spontaneous decay of compound I upon its formation, which make the investigation of compound I much more difficult than those of other peroxidases. With canine myeloperoxidase, a 40-fold excess of hydrogen peroxide was mixed with the enzyme yielding a relatively pure compound I, which has a half-life of about 100 ms (9). With at least 25-fold excess of hydrogen peroxide, an approximate spectrum of compound I

for leucocyte myeloperoxidase was constructed by extrapolating stopped-flow data to zero time, and the half-life for the compound I was estimated to be 230 ms (11).

Different mechanisms have been proposed to account for the peculiar behavior of compound I. A natural assumption is that there may be some impurities carried over from enzyme preparation which are responsible for the spontaneous reduction of compound I to compound II, but this possibility as a major pathway for compound I reduction was excluded by an experiment in which the repeated titration with hydrogen peroxide did not improve the stability of compound I (12).

Iwamoto et al. (12) ascribed the abnormal stoichiometry of the reaction between myeloperoxidase and hydrogen peroxide to the "true catalase activity" of myeloperoxidase, i.e., after compound I formation it reacts with more hydrogen peroxide and returns to the native state with concomittant evolution of molecular oxygen:

$$MPO + H_2O_2 \longrightarrow MPO\text{-}I + H_2O$$

$$MPO\text{-}I + H_2O_2 \longrightarrow MPO + O_2 + H_2O$$

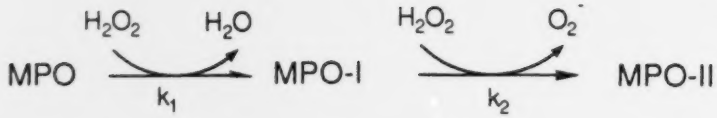
Their major evidence was the release of oxygen from the MPO/H_2O_2 system detected by an oxygen electrode. Although this mechanism provides an

explanation to the abnormal stoichiometry of compound I formation, it does not appear that the catalatic reaction is a dominant pathway for the following reasons. As the authors estimated, the catalatic reaction is even faster than compound I formation. Then there would be a rapidly established steady state at which the ratio [MPO-I]/[MPO] would be a constant independent of the initial concentration of hydrogen peroxide. One would predict that varying the initial concentration of hydrogen peroxide would not have much effect on the yield of compound I. However, this is not consistent with the results from the present study. Also, if the catalatic breakdown of hydrogen peroxide to oxygen were the major reaction in the presence of myeloperoxidase, oxygen bubble formation would be evident in the presence of a large excess of hydrogen peroxide. This is not observed. Furthermore, this mechanism gives no account for the spontaneous reduction of compound I to compound II, which is a major factor contributing to the abnormal stoichiometry of compound I formation.

Is it possible that the instability of compound I and the biphasic nature of compound I conversion to compound II are caused by a difference in the reactivity of the two hemes in myeloperoxidase which results in the first formation of compound I in one monomer followed by its reduction by another monomer? Or could it be that there is a certain degree of negative cooperativity? These possibilities are also unlikely considering the reported comparative studies on the native dimeric MPO and hemi-MPO. These studies

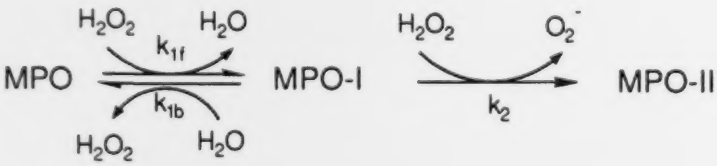
have shown that (i) the hemi-MPO and the dimeric MPO have identical visible spectra under either oxidized or reduced conditions, suggesting the same heme environments (2), (ii) the hemi-MPO and the native MPO show the same activity in both a Cl^- independent peroxidase assay and a Cl^- dependent bactericidal assay (2,27), and (iii) the yield and the rate of the compound II formation from the native enzyme and hydrogen peroxide are the same for the hemi-MPO and the dimeric MPO (12).

In the present study, three lines of experimental results are presented: (i) the complete formation of compound I from the native enzyme requires a large excess of hydrogen peroxide; (ii) the conversion of compound I to compound II is also hydrogen peroxide dependent and its biphasic time course becomes monophasic as the concentration of hydrogen peroxide increases, and (iii) superoxide dismutase enhances the conversion of compound I to compound II and turns the biphasic conversion into a faster monophasic process, presumably through the removal of superoxide generated in the MPO/H_2O_2 system. The ideal mechanism for the reactions of myeloperoxidase with hydrogen peroxide must be capable of accommodating these results. Scheme 1 represents the simplest mechanism for the reactions of myeloperoxidase with hydrogen peroxide, which takes into consideration the fact that both the formation of compound I from the



Scheme 1

native enzyme and the reduction of compound I to compound II are dependent on hydrogen peroxide. This scheme would predict a maximum in the time course of compound I concentration. However, this maximum yield of compound I, which is solely determined by the ratio of k_1 and k_2 when hydrogen peroxide is in a large excess, would not be affected by the change in hydrogen peroxide concentration. This is in contradiction with the observed hydrogen peroxide dependence of compound I yield, i.e., the higher the concentration of hydrogen peroxide, the greater the yield of compound I. The incorporation of an equilibrium for the formation of compound I, which would help to eliminate the contradiction, leads to scheme 2:



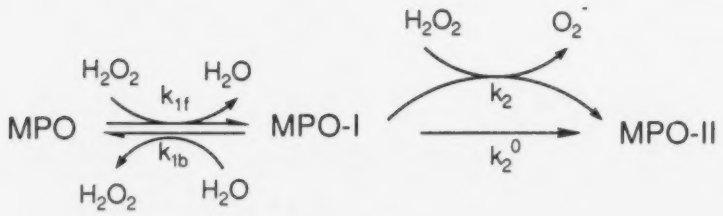
Scheme 2

This scheme, which was also proposed in previous studies (13,14), is more successful in that an explanation is provided for the abnormal

stoichiometry of compound I formation. With too little hydrogen peroxide the first equilibrium will not go far enough to the right, hence compound I formation will be incomplete. On the other hand, this incompletely formed compound I is subject to further reaction with hydrogen peroxide. Therefore the spectra we recorded are either those of a mixture of compound I and native enzyme, or of compound I and compound II. In other words, at no time could we observe absolutely pure compound I. In the case of a large excess of hydrogen peroxide, however, the first equilibrium lies much further to the right so that compound I is almost fully formed before it is further reduced to compound II by hydrogen peroxide, enabling us to record the spectra of relatively pure compound I.

One difficulty with scheme 2 is that it cannot provide an account for the biphasic nature of the conversion from compound I to compound II. Under the experimental conditions in this study, the equilibrium in the first step is reached rapidly (within 50 msec) while the conversion of compound I to compound II is a much slower process and lasts for a few seconds. Previous studies leading to this scheme also showed that $k_{1f}[H_2O_2] >> k_{1b} >> k_2[H_2O_2]$ (13,14). Theoretical analysis under such conditions would predict a monophasic time course for the conversion of compound I to compound II. However, this conversion has two distinct phases, which characterize both the decay of compound I and the formation of compound II.

While hydrogen peroxide is shown to be a driving force for the reduction of compound I to compound II, the autodecay of compound I, in which an intramolecular electron transfer is probably responsible for the reduction of compound I, may not be excluded. Does this pathway, together with the reduction of compound I by hydrogen peroxide (scheme 3), make the conversion of compound I to compound II biphasic? A negative answer is

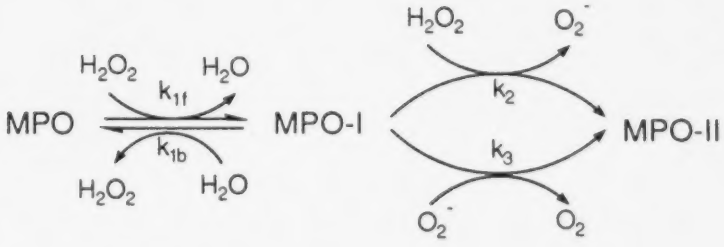


Scheme 3

obtained from a kinetic analysis of the reactions depicted in scheme 3, which again predicts a monophasic conversion of compound I to compound II when hydrogen peroxide is in excess. The pseudo-first order rate constant characterizing this monophasic process would be the sum of $k_2 [H_2O_2]$ and k_2^0.

Superoxide is generated from the one-electron oxidation of hydrogen peroxide. Cytochrome c added to the MPO/H_2O_2 system was found to be reduced, presumably by superoxide generated therein (13). It is unlikely that

superoxide stays as a mere spectator in the conversion of compound I to compound II. Instead, the role of superoxide as a reducing agent other than hydrogen peroxide in the conversion of compound I to compound II should also be considered. This consideration leads to scheme 4, in which both hydrogen peroxide and superoxide contribute to the reduction of compound I:



Scheme 4

With two different reducing substrates proposed to participate in the reduction of compound I to compound II, scheme 4, at first glance, was thought to be capable explaining the biphasic kinetics of compound I reduction. However, this was proved wrong in a further examination, which is outlined as below.

(i) If $k_3 > k_2$, the second reaction would not constitute a slower phase since this reaction would not retard the conversion of MPO-I to MPO-II. On the other hand, the second step cannot be faster either because it is limited by the generation of O_2^- in the first step.

(ii) If $k_3 \leq k_2$, the contribution of the second reaction to the MPO-I --> MPO-II conversion would be negligible. Under every circumstance $[O_2^{\cdot-}] < [MPO]_0$, but $[H_2O_2] >> [MPO]_0$ in all our experiments, thus $[O_2^{\cdot-}] << [H_2O_2]$. The combination of $k_3 \leq k_2$ and $[O_2^{\cdot-}] << [H_2O_2]$ would make the second reaction unimportant.

In addition, the conversion of biphasic kinetics to monophasic kinetics for the reduction of compound I to compound II by addition of SOD presents another challenge to the mechanism shown in scheme 4. This effect, which appears to be associated with the biphasic nature of compound I reduction to compound II, requires another mechanism in which the role of superoxide in the conversion of compound I to compound II needs to be redefined.

The inactivation of heme enzymes including peroxidases by superoxide in the presence of excess hydrogen peroxide has been reported previously (28,29). There is a widespread belief that a superoxide-driven Fenton reaction or an iron-catalyzed Haber-Weiss reaction gives rise to a powerful oxidant, •OH. However, this is a very controversial aera. It has been postulated that •OH generated from such reactions can attack the porphyrin moiety, leading to the inactivation of the enzymes (30). This superoxide-mediated inactivation, if present in the MPO/H_2O_2 system in this study, may turn an otherwise monophasic reduction of compound I into a biphasic process, with the slower phase controlled by the inactivation of the enzyme.

The inactivation of enzyme involving superoxide could also provide an explanation to the experimental finding that SOD speeds up the reduction of compound I to compound II and converts the biphasic process to monophasic. However, this hypothesis contradicts the experimental results in that it would predict a stronger inactivation of the enzyme at a higher hydrogen peroxide concentration. On the other hand, the •OH-induced inactivation of the enzyme is supposed to be irreversible, thus the recovery of the enzyme would be rapidly reduced with the repetition of H_2O_2-driven enzymatic cycle. This is also contradictory to a previously reported finding that the the enzyme maintained a high level of recovery after three H_2O_2-driven enzymatic cycles (12).

The search for a mechanism accounting for the behavior of superoxide in its interaction with compound I has been shed some light by a very recent study, in which halide binding to MPO-CN was studied as a model for substrate binding to compound I of myeloperoxidase (31). EPR evidence for the binding between halide and MPO-CN was obtained at very low temperature (10 K) and relatively high enzyme concentrations (200 - 400 μM). It appears that halide binds to an amino acid residue in the distal heme cavity of MPO-CN. The distal histidine and arginine, the key residues in controlling activity of many peroxidases, are the most probable candidate residues. Though the binding is believed to occur over a wide pH range, only at acidic

pH is direct contact between halide and the ferryl oxygen of the enzyme allowed, which is presumably mediated by the binding of halide ion to the distal histidine. As the consequence of such direct contact between halide and ferryl oxygen, the electronic structure of heme iron is modulated hence small EPR spectral changes were detected. At higher pH, the deprotonation of the histidine may result in the binding of halide to a residue other than the distal histidine, possibly the distal arginine or an amino acid further away from the ferryl oxygen, and mediate a long range effect on the iron center. Since the binding at higher pH causes no direct contact between the halide ion and the ferryl oxygen, no spectral changes have been detected. Because the cyanide complex of MPO often serves as a structural model of compound I, it is suggested that the structural basis of substrate halide binding to compound I is similar to that of the halide binding to MPO-CN. This is supported by kinetic studies showing that the rate of halide oxidation by MPO-I reaches a maximum at acidic pH but drops at pH > 5 (32).

The results and suggestions from the work described above have inspired us to reconsider the interaction of superoxide with compound I in the present study. The reduction of compound I by superoxide is not necessarily a simple one-electron jump process. Instead, it may proceed through an initial

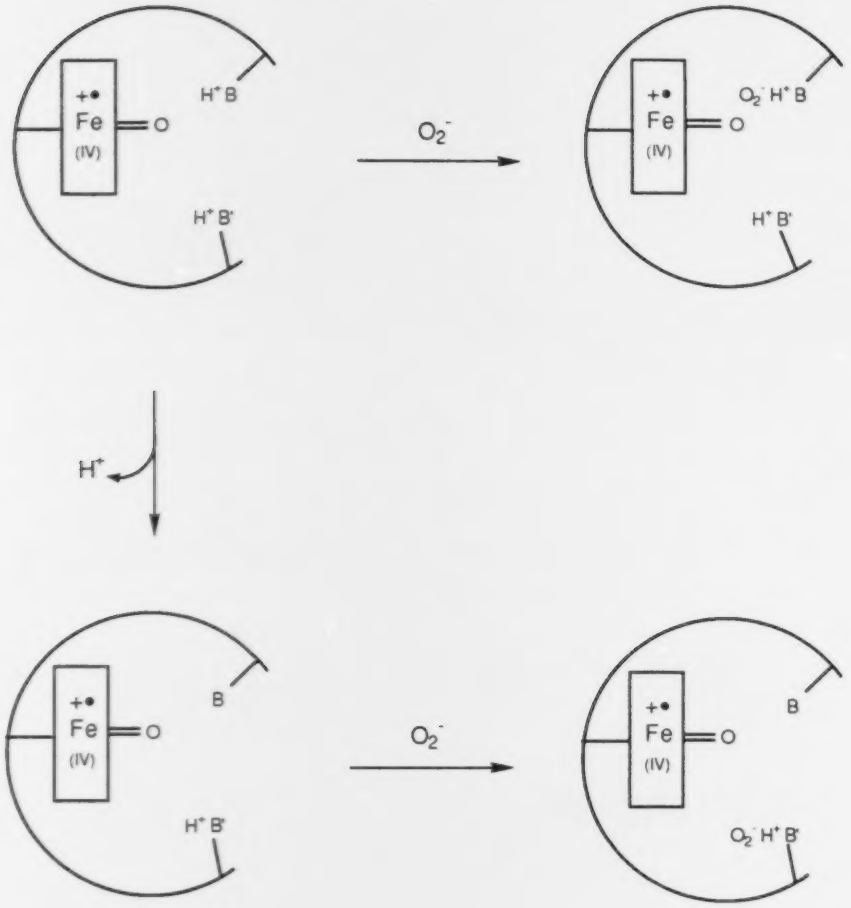
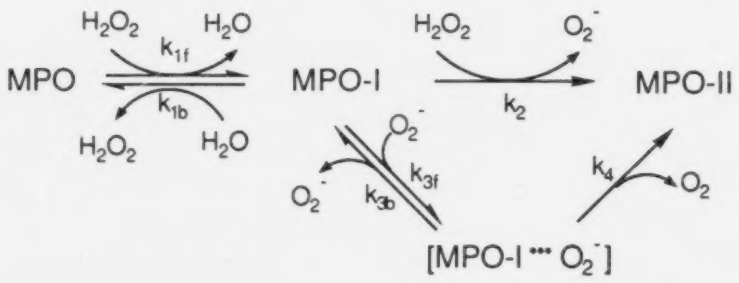


Figure 6.8. Putative scheme depicting superoxide binding to MPO-I. B and B' denote functional groups of distal amino acids.

binding of superoxide to compound I (Fig. 6.8) followed by the conversion of the complex to compound II and oxygen. Assuming that the binding sites in compound I are similar for halide and superoxide and considering that the experiments in this study were not performed at acidic pH, we suggest that superoxide may bind to an amino acid (HB' in Fig. 6.8) somewhat distant from the ferryl oxygen and initiate a protein-mediated electron transfer to the iron center of compound I. The binding does not lead to the direct contact between superoxide and the ferryl oxygen, hence no significant spectral changes can be observed but the overall reaction between compound I and superoxide would be slower than expected on the basis of a simple electron jump mechanism.

Based on the consideration given above, scheme 5 is proposed for the reactions involved in the MPO/H_2O_2 system under the experimental conditions in this study:



Scheme 5

There are three predictions which can be drawn from this scheme:

(1) An equilibrium exists between the native enzyme and compound I, which can be shifted by varying the concentration of hydrogen peroxide.

(2) A major driving force of the conversion of compound I to compound II under the current condition is hydrogen peroxide, which not only acts as an oxidizing substrate toward ferric enzyme but also plays a role of reducing substrate toward compound I.

(3) Superoxide is generated as the product of the reduction of compound I by H_2O_2. It is also involved in the compound I reduction through a fast binding, followed by a slower decay to yield compound II.

Some puzzles around myeloperoxidase behavior, either previously reported or presently observed, can be rationalized in the light of this mechanism as illustrated below.

There are two interesting features associated with the time courses for the conversion of compound I to compound II: (i) its biphasic nature, and (ii) its hydrogen peroxide concentration dependence. According to our mechanism, the reduction of compound I to compound II is mainly dependent upon hydrogen peroxide but also upon superoxide generated from the oxidation of hydrogen peroxide. Hydrogen peroxide directly reduces compound I, giving a faster phase of the time courses. Superoxide, on the other hand, once generated from hydrogen peroxide oxidized by compound I, rapidly binds to

compound I and then slowly converts compound I to compound II, causinging a slower reaction. Thus biphasic time courses are expected. However, a high concentration of hydrogen peroxide favors the direct reduction of compound I by hydrogen peroxide. Therefore, a convergence of the time courses to one faster phase should be observed with the increasing hydrogen peroxide concentration. This is confirmed by the results shown in Fig. 6.3 and Fig. 6.4.

The proposed interference of superoxide with the reduction of compound I by hydrogen peroxide can be prevented by adding superoxide dismutase to the system. Since the the inactivation and/or reduction of SOD some sources by hydrogen peroxide were reported (33-35), two different types of SOD, Cu/Zn SOD from bovine erythrocytes and MnSOD from *E. coli* , were used in this study. The reported rate constants for the inactivation and/or reduction by hydrogen peroxide are so low that it is virtually impossible for the products of these reactions to alter the time course of compound I reduction significantly. However, the inactivation and/or reduction of SOD inevitably lowers its efficiency in superoxide removal. This, on the other hand, may help to explain why a relatively high concentration of SOD is needed to completely eliminate the proposed interference of superoxide.

Oxygen evolution is also suggested by this mechanism, only at a lower level, and by a different mechanism than that suggested by Iwamoto et al. (12). The release of molecular oxygen can be considered as the result of the

involvement of superoxide in compound I reduction. Also, when the observation period is long enough, more oxygen may be generated by the oxidation of superoxide by compound II:

$$\text{compound II} + O_2^- \longrightarrow \text{native enzyme} + O_2$$

The regeneration of native enzyme initiates another enzyme cycle, hence more oxygen evolution. The reduction of compound II by superoxide, which was previously proposed for lactoperoxidase (36) , is not included in the mechanism we propose in this study. The reason is that the reaction must be much slower than all others included in our mechanism so that its role would be negligible under our experimental conditions. Evidence can be found from the time courses for compound II formation recorded at the isosbestic point between compound I and native enzyme. The monotonic increase of compound II concentration indicates that under our experimental conditions there is no significant conversion of compound II to the native state.

Also in regard to the previously proposed catalase activity of myeloperoxidase, it is interesting that the proposal, which does not account for compound II as an intermediate, cannot explain the observed enzyme cycling (native state → compound I → compound II →native state •••) with the repeated additions of hydrogen peroxide (12). With the mechanism proposed

in this study, however, it is easy to understand why hydrogen peroxide alone is enough to drive myeloperoxidase through a complete enzyme cycle.

In conclusion, we have investigated the formation of myeloperoxidase compound I and its conversion to compound II in the presence of hydrogen peroxide. At least a 100-fold excess of hydrogen peroxide was used to obtain relatively pure compound I. The time courses for the conversion of compound I to compound II are biphasic in nature and also dependent on hydrogen peroxide concentration. Either addition of sufficient hydrogen peroxide or of sufficient superoxide dimutase to the system can convert the biphasic reactions to monophasic. A new mechansism is developed, in which the abnormal stoichiometry of the formation and the spontaneous decay of compound I are explained by the roles of both hydrogen peroxide and superoxide as reducing substrates in the reduction of compound I to compound II.

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CHAPTER SEVEN

QUANTITATIVE STUDIES ON MPO REACTIONS WITH HYDROGEN PEROXIDE*

7.1 SUMMARY

The kinetics of the conversion of myeloperoxidase to its compounds I and II by hydrogen peroxide has been studied using a combination of calculations based upon stopped-flow measurements and simulations. For the initial step, rate constants were obtained under pseudo-first order conditions: $(2.8 \pm 0.5) \times 10^7$ M^{-1} s^{-1} for the formation of compound I from native enzyme and hydrogen peroxide, and (20 ± 4) s^{-1} for the reverse reaction, namely the decomposition of compound I to native enzyme and hydrogen peroxide. Compound I, once formed, also reacts with hydrogen peroxide to form compound II and superoxide. The second order rate constant for this reaction was measured in the presence of superoxide dismutase and a value of $(1.6 \pm 0.1) \times 10^5$ M^{-1} s^{-1} was obtained. We proposed in the previous chapter that superoxide, produced in the reduction of compound I by hydrogen peroxide, also reduces compound I to compound II through an intermediate complex. Relevant rate constants were obtained

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* Abbreviations: MPO, myeloperoxidase(EC 1.11.1.7 donor-H_2O_2 oxidoreductase); MPO-I, myeloperoxidase compound I; MPO-II, myeloperoxidase compound II; SOD, superoxide dismutase.

through parameter optimization on the basis of digital solutions of differential rate equations. They are 2.5 x 10^9 M^{-1} s^{-1} and 100 s^{-1} for the formation and the dissociation of compound I-superoxide complex, and 0.1 s^{-1} for the conversion of compound I-superoxide complex to compound II and molecular oxygen. The overall kinetics predicted in terms of these quantitative results is consistent with the kinetic behavior of the MPO/H_2O_2 system, providing a support to the mechanism we proposed in the previous chapter.

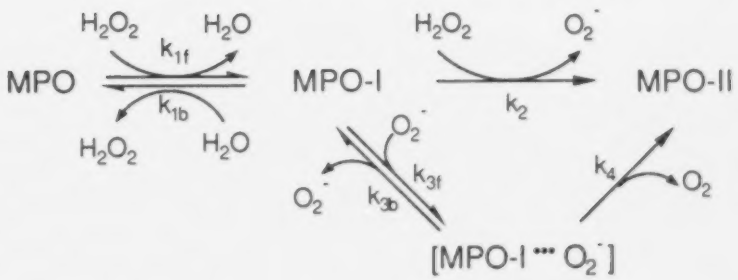
7.2 INTRODUCTION

Myeloperoxidase is a heme-containing mammalian enzyme located in polymorphonuclear leucocytes (1). The enzyme plays a significant physiological role in the body's defense against microbial infection (2). As a peroxidase, it undergoes the normal peroxidatic cycle which involves its three spectroscopically distinguishable forms: native enzyme, compound I and compound II. The native enzyme reacts with hydrogen peroxide to form compound I, which in turn reacts with electron donors resulting in the formation of compound II. Compound II can also react with electron donors to regenerate native enzyme and complete an enzyme cycle (3,4).

The oxidized enzyme intermediate directly involved in the anti-microbial mechanism of myeloperoxidase is compound I , which converts chloride ion into hypochlorous acid, an *in vivo* bactericidal agent (5,6).

Though the conversion of myeloperoxidase to its oxidized intermediates has been widely studied, there are still some mechanistic puzzles. Difficulties have been encountered in preparing pure and stable oxidized intermediates, especially compound I. Previous studies revealed that large excess of hydrogen peroxide is required to achieve the complete formation of compound I, though the stoichiometries reported up to now appear inconsistent with each other (7-9).

In chapter six, a new mechanistic scheme has been proposed for the reactions involved in the MPO/H_2O_2 system under the experimental conditions in this study:



Scheme 1

In scheme 1, k_{1f} refers to the second order forward reaction for the formation of MPO-I and k_{1b} the first order back reaction for the dissociation of MPO-I into MPO and H_2O_2; k_2 is the second order rate constant for the reduction of MPO-I to MPO-II by H_2O_2 ; k_{3f} and k_{3b} are the rate constants for the formation and the dissociation of the complex, and k_4 is the rate constant for conversion of the MPO-I•••O_2^- complex to MPO-II and O_2 .

The success of this model in the qualitative explanation of the behavior of the MPO/H_2O_2 system suggests that these reactions predominate in the conversion of native myeloperoxidase to compound II via compound I in the absence of exogenous electron donors.

In this chapter, the results of a quantitative treatment of the MPO/H_2O_2 system based upon this model will be presented. With the rate constants involved in the model being either determined by measurements or estimated from simulations, calculations were made on the overall kinetics of the conversion of native enzyme to compound II via compound I, which is in agreement with the experimental results obtained in both the previous and the present chapters.

7.3 MATERIALS AND METHODS

Materials

Myeloperoxidase was isolated from bovine spleens and further purified using a combination of published procedure and some minor modifications (10-12). The enzyme preparations used in this study have RZ number of 0.80 or higher. The concentration of myeloperoxidase was determined spectrophotometrically at 430 nm with a molar absorptivity of 1.78×10^5 M^{-1} cm^{-1} (13).

Cu/Zn SOD from bovine erythrocytes was obtained from Sigma as crystals. The enzyme was dissolved prior to use and the concentration was checked spectrophotometrically using a molar absorptivity of 15.9 mM^{-1} cm^{-1} at 265 nm (14).

30% hydrogen peroxide solution was purchased from BDH Chemicals. The concentration of the solution used in experiments was determined in assays employing the oxidation of iodide catalyzed by horseradish peroxidase (15). Chemicals used for the buffers were of reagent grade and used without further purification. All solutions were prepared from water deionized by a Milli-Q purification system.

Stopped-flow Kinetic Experiments

Stopped-flow experiments were performed on a Photal Rapid Reaction Analyzer Model RA-601 equipped with a 1-cm observation cell. Rate constants for the experimental traces obtained in stopped-flow experiments were determined by nonlinear least-squares analysis, carried out by an Alps Electric Co. Model No. CP10A computer interfaced to the apparatus.

All experiments were performed at 25.0 ± 0.5^0 C and an ionic strength of 0.1 M due entirely to the contribution of the buffer. A final concentration of 0.4 μM for myeloperoxidase was used throughout the study while the concentration of hydrogen peroxide varied from 6 to 100 μM in order to ensure pseudo-first order conditions.

The formation of compound I was monitored at 429 nm, the isosbestic point between compound I and compound II with a band pass of 3.5 nm. In a typical experiment 2 mL of buffered native enzyme solution was placed in one reservior of the stopped-flow apparatus and 2 mL of buffered hydrogen peroxide solution in another.

The kinetics of compound I reduction by hydrogen peroxide was studied in the system consisting of myeloperoxidase and large excesses of hydrogen peroxide (100 fold or larger) in the presence of superoxide dismutase. Though smaller excesses of hydrogen peroxide are usually enough for a pseudo-first order kinetic study, large excesses of hydrogen

peroxide were used here to assure relatively complete formation of compound I before its reduction. With the addition of superoxide dismutase the interference of superoxide with the reaction between compound I and hydrogen peroxide was removed so that hydrogen peroxide was the only reducing substrate. Time courses for both the decay of compound I and the formation of compound II were recorded. The former was monitored at 442 nm, the isosbestic point between the native state and compound II, and the latter was monitored at 456 nm, the isosbestic point between the native state and compound I. The solution containing both myeloperoxidase and superoxide dismutase was placed in one reservoir and hydrogen peroxide solution in another. The alternative arrangement, in which the solution containing only myeloperoxidase is placed in one reservior and the mixture of hydrogen peroxide and superoxide dismutase in another, was not adopted because of the slow reduction and inactivation of superoxide dismutase by hydrogen peroxide (16-18).

Computer Simulations

While we can inhibit the reaction between compound I and superoxide by using SOD so that the reduction of compound I by hydrogen peroxide can be studied independently, it is impossible to study the reduction of compound I by superoxide independently from that by hydrogen peroxide since the latter

is the prerequisite for the formation of the former. Thus simulations were calculated to estimate the rate constants for superoxide reacting with compound I.

For the mechanistic model shown in scheme I, the analytical solution of the transient kinetics is impossible. Thus computer programs were constructed to solve the differential rate equations digitally. The solutions give the time course of concentration change for each enzyme and substrate species. With the rate constants for compound I formation and for compound I reacting with hydrogen peroxide being measured in stopped-flow experiments, the digital solutions for the concentration time courses are dependent on the rate constants of superoxide reacting with compound I. Thus by simulating the experimentally recorded concentration time courses of different enzyme intermediates under different conditions, values of the rate constants for compound I reacting with superoxide were estimated (see Appendix I).

7.4 RESULTS

Formation of Compound I

Under the condition of large excesses of hydrogen peroxide, the pseudo-first order rate consatnt, $k_{1,obs}$, for the reversible reaction between

myeloperoxidase and hydrogen peroxide was obtained from the exponential time-courses. The first order kinetics are described by

$$- d[MPO] / dt = k_{1,obs} [MPO]$$

where

$$k_{1,obs} = k_{1f} [H_2O_2] + k_{1b}$$

The pseudo-first order rate constant $k_{1,obs}$ is plotted versus hydrogen peroxide concentration in Fig. 7.1. The inset to Fig. 7.1 shows a typical stopped-flow trace for compound I formation displaying monophasic exponential character. The forward second order rate constant, obtained from the slope of the plot, k_{1f}, is $(2.8 \pm 0.5) \times 10^7$ M^{-1} s^{-1}, and the rate constant for the reverse reaction, k_{1b}, obtained from the intercept of the plot, is (20 ± 4) s^{-1}.

Reduction of Compound I by Hydrogen Peroxide

With hydrogen peroxide in excess (100 fold and larger) and sufficient superoxide dismutase present, the reduction of compound I or the formation of compound II displays monophasic exponential traces. Pseudo-first order data treatment was applied to both compound I decay and compound II

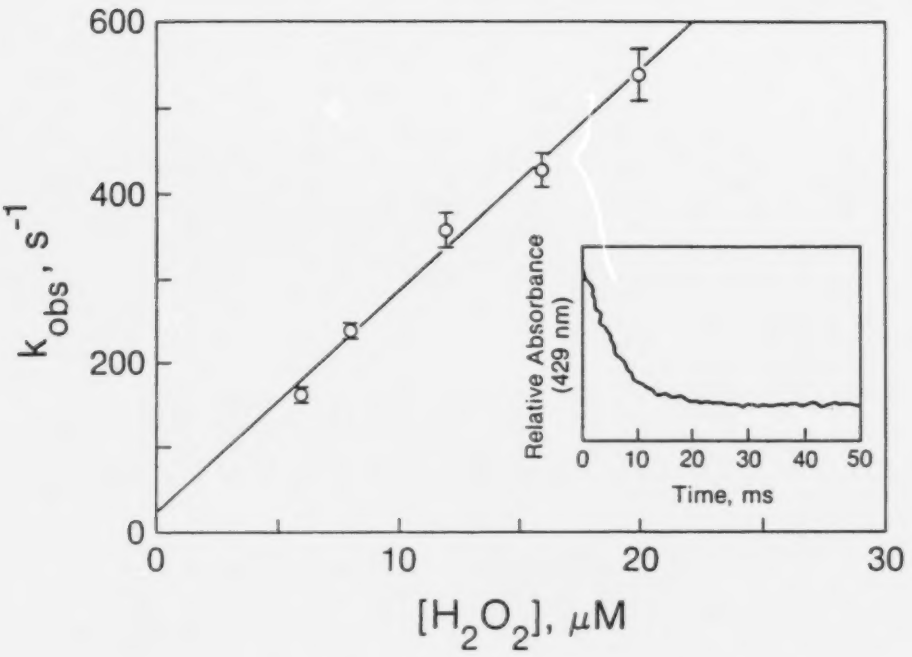


Figure 7.1. Pseudo-first order rate constants for compound I formation plotted against $[H_2O_2]$. Slope: $(2.8 \pm 0.5) \times 10^7$ M^{-1} s^{-1}; Intercept: (20 ± 4) s^{-1}. Inset shows a typical exponential trace of the reaction at 429 nm. Final enzyme concentration: 0.4 μM MPO in 0.1 M pH 7.0 phosphate buffer.

formation, and the rate constants obtained in the two cases turned out to be the same within the experimental error. We choose to present the results for compound II formation, which have smaller experimental errors mainly because the absorbance changes at 456 nm where we observe compound II formation are larger than those at 442 nm where we observe compound I decay. In Fig. 7.2, the pseudo-first order rate constant, $k_{2,obs}$, is plotted versus hydrogen peroxide concentration, and a straight line with a small intercept was obtained which can be described by

$$k_{2,obs} = k_2 [H_2O_2] + k_2^0$$

with $k_2 = (1.6 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ and $k_2^0 = (0.5 \pm 0.2)\ s^{-1}$, where k_2 is the second order rate constant for the reaction between MPO-I and hydrogen peroxide and k_2^0 represents the spontaneous decomposition of MPO-I to MPO-II. Thus hydrogen peroxide is mainly responsible for the reduction of compound I under our experimental condition, but there seems to be some spontaneous decay of MPO-I to MPO-II.

Reduction of Compound I by Superoxide

According to the computer calculations based upon Scheme I, the reaction between compound I and superoxide proceeds via a fast formation

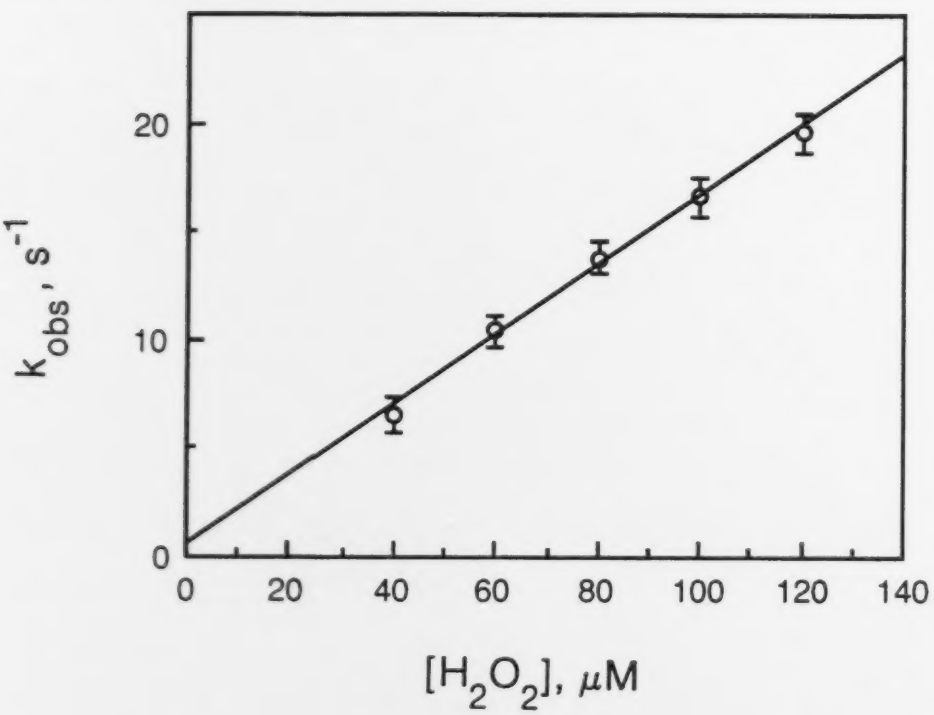


Figure 7.2. Plot of k_{obs} vs $[H_2O_2]$ for the formation of compound II from compound I and hydrogen peroxide. Slope: $(1.6 \pm 0.1) \times 10^5$ M^{-1} s^{-1}; Intercept: (0.5 ± 0.2) s^{-1}. Final concentrations: 0.4 μM MPO and 5 μM SOD in 0.1 M pH 7.0 phosphate buffer.

of enzyme-substrate complex followed by a slow breakdown of the complex to give compound II. The relevant rate constants estimated from the simulations are $k_{3f} = 2.5 \times 10^9\ M^{-1}\ s^{-1}$ for the formation and $k_{3f} = 100\ s^{-1}$ for the dissociation of the MPO-I•••O_2^- complex, and $k_4 = 0.1\ s^{-1}$ for the conversion of the complex to compound II.

To test the efficacy of our mechanistic model in accounting for the behavior of MPO/H_2O_2 system and the accuracy of the estimated rate constants, time courses for compound I formation, compound I decay and compound II formation were calculated based on the model and the rate constants obtained in this study. Comparisons are made in Fig. 7.3 and Fig. 7.4 between the measured and the calculated time courses of compound I decay and compound II formation for different hydrogen peroxide concentrations. There is a good agreement between the predicted and the real time courses.

7.5 DISCUSSION

The apparent second order rate constant for bovine spleen myeloperoxidase compound I formation is determined to be $(2.8 \pm 0.5) \times 10^7\ M^{-1}\ s^{-1}$ in this study, which is comparable to the values previously reported: $(1.0 \pm 0.2) \times 10^7\ M^{-1}\ s^{-1}$ for rat chloroma myeloperoxidase (19); 2.0×10^7

M^{-1} s^{-1} for canine pus myeloperoxidase (20); and 2.3 x 10^7 M^{-1} s^{-1} for human granulocyte myeloperoxidase (9). This is also the same order of magnitude as that of compound I formation of horseradish peroxidase: (1.8 ± 0.4) x 10^7 M^{-1} s^{-1} (21).

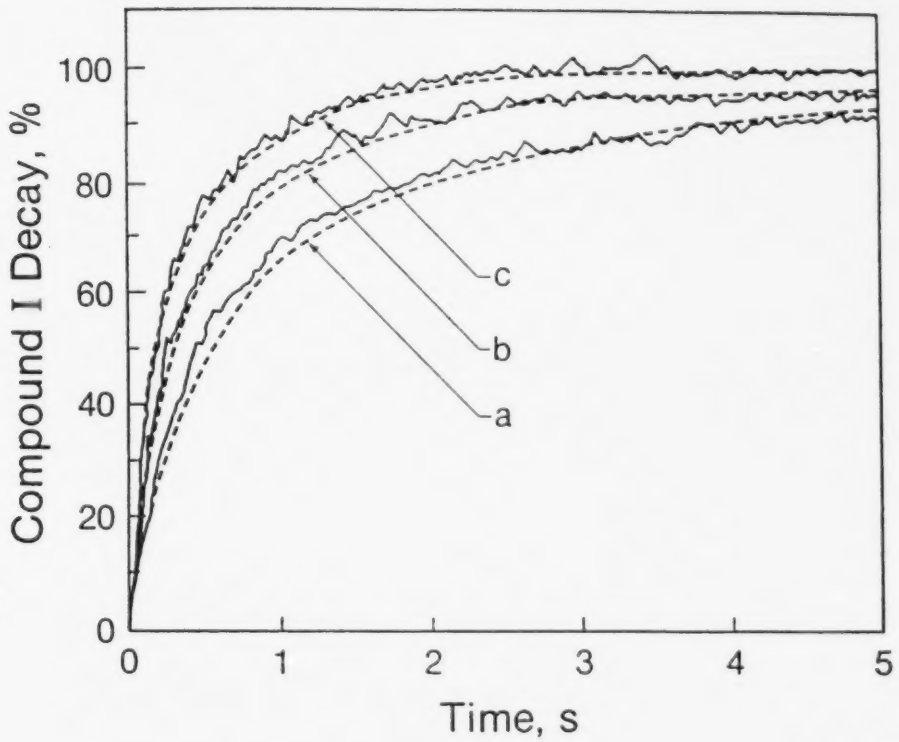


Figure 7.3. Comparison between the measured and the calculated time courses of compound I decay for different H_2O_2 concentrations. For the convenience of comparison, the measured time courses were expressed in percentage of compound I decay, which were obtained from the time courses of absorbance change through calibration. Absorbance change was measured at 442 nm which reflects the change of the total concentration of compound I, i. e., both the free and the superoxide-bound forms were accounted, since the binding of superoxide makes no difference in the Soret absorption of compound I as indicated in chapter six. Enzyme concentration: 0.4 μM. H_2O_2 concentrations: 8 μM (a), 20 μM (b), and 40 μM (c).

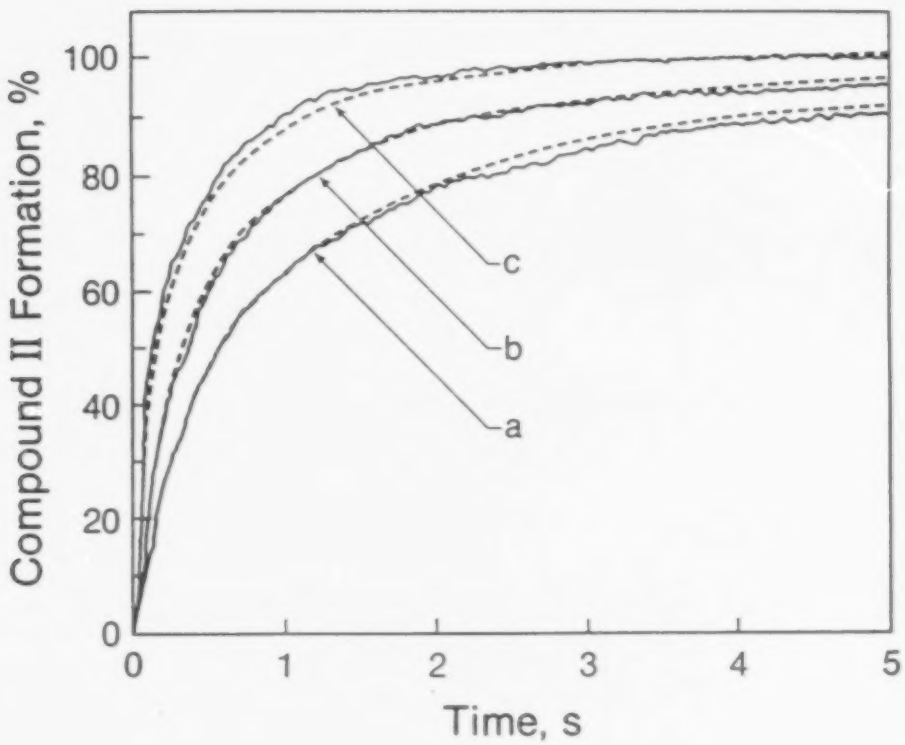


Figure 7.4. Comparison between the measured and the calculated time courses of compound II formation for different H_2O_2 concentrations. The measured time courses were expressed in percentage of compound II formation, which were obtained from the corresponding time courses of absorbance change through calibration. Measurements of absorbance change were performed at 456 nm. Enzyme concentration: 0.4 μM. H_2O_2 concentrations: 8 μM (a), 20 μM (b), and 40 μM (c).

The finite intercept in Fig. 7.1 is interpreted as the first order rate constant for the reverse reaction of compound I formation, the decomposition of compound I to the native enzyme and hydrogen peroxide. In the study of leucocyte myeloperoxidase compound I formation, a Scatchard plot was constructed using a wide range of hydrogen peroxide concentrations. Experimental details for the construction of this plot are not given. The plot yielded a dissociation rate constant for compound I of 1.7 μM (9), from which one can calculate a dissociation rate constant of 39 s^{-1}, about double the value of our result. The possibility of reversal of compound I formation has also been discussed on the basis of redox potentials (22).

With all the rate constants for our mechanistic model obtained in this study, a quantitative account for the abnormal stoichiometry of compound I formation and the instability of compound I becomes possible. Predicted on the basis of our model, the increase of hydrogen peroxide concentration leads to the increase in the yield and the decrease in the stability of compound I (Fig. 7.5). In our calculations the yield of compound I formation is expressed by the ratio of the maximum concentration of compound I to the initial concentration of native myeloperoxidase and the stability of compound I is expressed by the time interval between the point when compound I reaches its maximum concentration and the point when 10% of this peak concentration has been lost. The use of different initial concentrations of

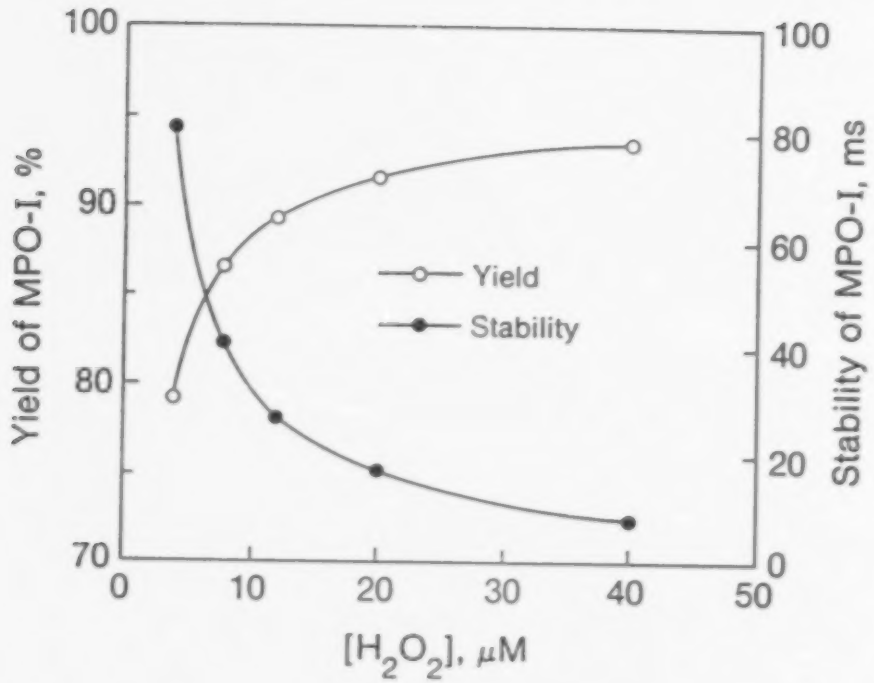


Figure 7.5. Predicted trends for the changes in the yield and the stability of compound I: effect of changes in H_2O_2 concentration. An initial concentration of 0.4 μM for native enzyme is assumed in the calculation. Yield of compound I formation is expressed by the ratio of the maximum concentration of compound I to the initial concentration of myeloperoxidase. Stability of compound I is expressed by the time interval between the point when compound I reaches its maximum concentration and the point when 10% of this peak concentration has been lost.

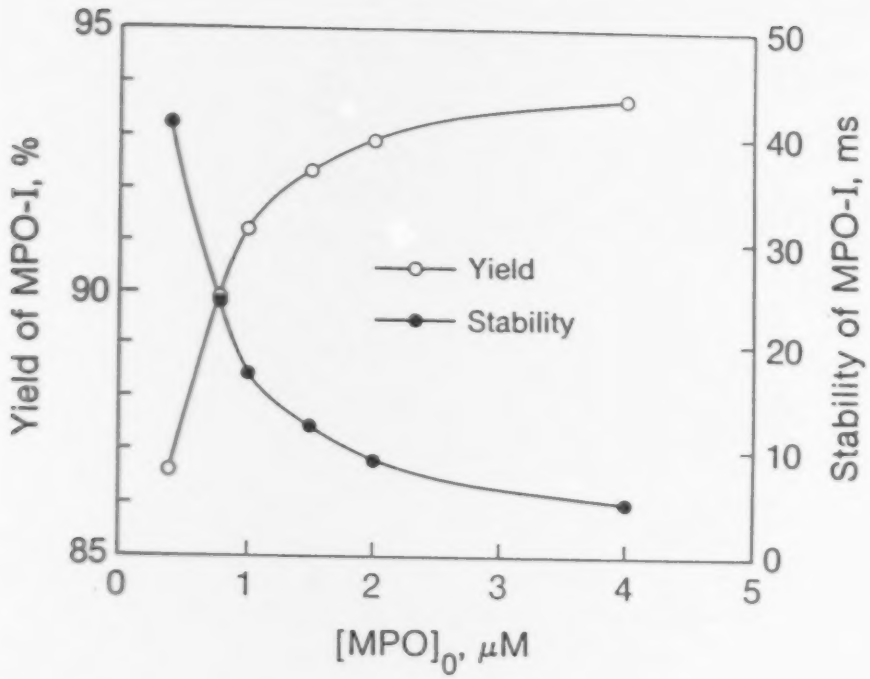


Figure 7.6. Predicted trends for the changes in the yield and the stability of compound I: effect of changes in initial native enzyme concentration. A 20-fold excess of hydrogen peroxide is assumed through the calculation. Yield and stability are expressed in the same way as described in Fig. 7.5.

myeloperoxidase will also result in different yield and stability of compound I, even though the ratio is maintained constant between the initial concentrations of hydrogen peroxide and the native enzyme (Fig. 7.6). This conclusion should be kept in mind when the reaction stoichiometries and stabilities observed in different studies are compared. The initial concentrations of the enzyme used in those studies may not be the same.

The reaction between compound I and hydrogen peroxide was studied previously with chloroperoxidase, and values of 2×10^5 M^{-1} s^{-1} (23) and 3.4×10^5 M^{-1} s^{-1} (24) for the second order rate constant were obtained. Though a different mechanism was proposed, which attributes the reaction to the catalatic activity of chloroperoxidase, the reported values for the second order rate constant are close to that of myeloperoxidase compound I reacting with hydrogen peroxide, 1.6×10^5 M^{-1} s^{-1} , obtained in this study.

The reduction of human leucocyte myeloperoxidase compound I by hydrogen peroxide was investigated previously by Hoogland et al. (25), and a second order rate constant of 3.5×10^4 M^{-1} s^{-1} was reported. A strict comparison of this value with ours is hampered by the lack of data on the standard deviation along with their results. The apparently lower rate constant obtained in their study may be related to the fact that the interference of superoxide with the reduction of compound I by hydrogen peroxide, which is observed in the present study, was not taken into their consideration. In

another study on bovine spleen myeloperoxidase, a second order rate constant of (8.1±0.1) x 10^4 M^{-1} s^{-1} was reported (26). However, this value should be examined with caution because it was obtained by approximately fitting biphasic time courses into monophasic curves, which may have resulted in a lower rate constant for the fast phase of the time courses.

Whereas the finite intercept in Fig. 7.1 is interpreted as the reverse reaction of compound I formation, Fig. 7.2 also shows a finite intercept, which is assigned to the autoreduction of compound I. Reversibility is not responsible for the intercept because one product of the forward reaction, superoxide, is scavenged upon its production (The rate constants for its production and scavenging are 1.6 x 10^5 M^{-1} s^{-1} and 1.6 x 10^9 M^{-1} s^{-1}, respectively) so that no reverse reaction can actually occur in the presence of adequate superoxide dismutase. The source of the electron in the minor pathway of compound I autoreduction needs some speculation. An intramolecular electron transfer may be responsible, which probably involves amino acid residue(s) of the protein moiety of the enzyme. Also, small amount of impurities in solutions may be involved. However, this should not be mixed up with the argument that the impurities in the samples are solely responsible for the conversion of compound I to compound II, which would suggest a decay of compound I independent of hydrogen peroxide concentration. The major role of reducing impurities in the reduction of compound I was also

excluded by Iwamoto et al. (27), who found that the repeated titration with hydrogen peroxide did not help to stabilize compound I of myeloperoxidase hence impurities can not be the major reducing force. Nevertheless, the possibility of the impurities as a minor reducing force should be considered, especially when enzyme concentration is low so that the level of impurities is not negligible.

Because the reduction of compound I by superoxide cannot be studied independently, the relevant rate constants, k_{3f} , k_{3b} and k_4 , cannot be measured directly. However, the values for these rate constants obtained from the simulations in this study can be compared to those of the reaction between superoxide and compound I of other peroxidases. In the case of MPO-I reacting with superoxide, since the equilibrium for the MPO-I$\bullet\bullet\bullet O_2^-$ complex formation is established relatively rapidly compared to the conversion of the complex to MPO-II and O_2 , the apparent second order rate constant for MPO-II formation, k_{app} , can be estimated from

$$k_{app} = k_4 k_{3f} / k_{3b}$$

(see Appendix II). Using the values obtained in this study for k_{3f} , k_{3b} and k_4 , k_{app} can be estimated to be 2.4×10^6 M^{-1} s^{-1}. This is of the same order of magnitude as that reported for the reduction of horseradish peroxidase

compound I by superoxide which was obtained in a pulse radiolysis study (28).

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CHAPTER EIGHT

GENERAL DISCUSSION

Since MPO was discovered fifty years ago, the enzyme has stimulated the interest of a steadily increasing number of scientists from different fields. Owing to the complexity of its structure and the uniqueness of its functions, many aspects regarding the mechanism of its reactions remain unsolved. It is anticipated that the enzyme will attract more attention in the future, considering its physiological significance which is evident from two basic facts: (i) it is present in mature neutrophils in extremely high concentration. As much as 2-5 % (w/w) of the total protein in these cells is MPO (1,2); (ii) while many mammalian enzymes have one or a few certain organ(s) as their major site of activity, MPO is ubiquitous in the body due to the transport of white blood cells.

The reaction of MPO with hydrogen peroxide is of primary importance. During phagocytosis hydrogen peroxide is readily availale to MPO (3) as the result of the respiratory burst in stimulated neutrophils (4,5). The reaction of MPO with hydrogen peroxide yields MPO-I, which is the only oxidized intermediate involved in both the two major catalytic activities of the enzyme: general peroxidase activity and halogenating enzyme activity.

Despite the crucial role of the reaction of MPO with hydrogen peroxide in the expression of both the two catalytic activities of the enzyme, the understanding of the reaction itself is far from adequate. This situation may result from the difficulty that the primary product of the reaction, MPO-I, is both unstable and reactive.

Two types of experiments, stopped-flow kinetic measurements and rapid scan spectral analyses, were employed to study the MPO/H_2O_2 system. New reaction pathways were postulated based on some qualitative observations and then further tested in a quantitative study. The combination of the qualitative and quantitative studies has led to a new mechanism, together with all the relevant kinetic parameters.

In the qualitative experiments, rapid scan spectral analyses indicate the abnormal stoichoimetry for the formation of MPO-I. A large excess of hydrogen peroxide is necessary to obtain relatively pure MPO-I, which may be attributed to the reversibility of MPO-I formation. Also, the higher the concentration of hydrogen peroxide the lower the stability of MPO-I. This observation strongly suggests the participation of hydrogen peroxide, most probably as a reducing substrate, in the decay of MPO-I. It is also observed that the rates of the decay of MPO-I and of the formation of MPO-II are characterized by a fast phase followed by a slow phase. The biphases converge to a faster monophase when the concentration of hydrogen peroxide is increased, or superoxide

dismutase (SOD) is added to the system. It appears that the conversion of MPO-I to MPO-II is more complicated than a simple reduction involving only hydrogen peroxide as the reducing agent. A new mechanism is postulated which provides explanations to all these observations, together with those reported previously. The central feature of this mechanism lies in the roles of hydrogen peroxide. MPO-I, formed from the oxidation of native enzyme by hydrogen peroxide is also reduced by hydrogen peroxide to form MPO-II and superoxide. Once formed, superoxide acts as another reducing substrate for MPO-I. While the abnormal stoichiometry for the formation of MPO-I and its subsequent decay can be accounted for by the role of hydrogen peroxide as both an oxidizing and a reducing substrate, the biphases observed for MPO-I decay may be attributed to the presence of two reductants: superoxide as well as hydrogen peroxide.

The quantitative study of the MPO/H_2O_2 system provides further evidence for the mechanism proposed on the basis of the qualitative study. Kinetic experiments in the presence of SOD show a linear dependence of the MPO-I decay on the concentration of hydrogen peroxide, confirming the role of hydrogen peroxide as a reducing substrate. SOD is used to prevent the reaction between MPO-I and hydrogen peroxide from the interference of superoxide, the product of the reaction.

The role of superoxide in the conversion of MPO-I to MPO-II in the presence of hydrogen peroxide is also defined in the mechanism proposed in this study. Once generated from the oxidation of hydrogen peroxide by MPO-I, superoxide can also reduce MPO-I to MPO-II throgh via a complex formation. The search of spectral evidence of the proposed MPO-I$\cdots O_2^-$ complex could be carried out in the future. In a relevant study reported recently, the binding of halide substrate to MPO-CN has been investigated (6). EPR spectral evidence has been found for the binding at very low temperature (10 K), relatively high enzyme concentrations (200-400 μM) and acidic pH. The binding is studied to provide a model for halide substrate binding to MPO-I. It is suggested that in different pH ranges halide binds to different amino acid residues in the distal heme cavity of MPO-I and only at acidic pH is the direct contact allowed between the bound halide and the feryl oxygen of compound I. We may learn from this example that (a) the reduction of MPO-I by small anions such as superoxide is not necessarily a simple one-step electron jump but may proceed through an initial binding to amino acid(s) followed by indirect electron transfer from the bound anion to heme iron center, and (b) spectral evidences for binding complexes of MPO-I are not always easy to detect in a certain range of pH or under conventional conditions (temperature, enzyme concentration, etc.). In our study low enzyme concentrations (< 1 μM) have to be used to keep the reaction slow enough for both the spectral

analysis and kinetic measurements. However, the low enzyme concentration inevitably limits the resolution of the spectra, especially in the case of compound I of peroxidases whose spectra themselves are featureless. Thus low enzyme concentration plus the featureless spectra of compound I makes the search for spectral evidence of MPO-I$\cdots O_2^-$ complex very difficult. Experiments performed at a higher enzyme concentration and a lower temperature would yield a higher spectral resolution while the rate of the reaction is still within a range suitable for both the spectral and the kinetic analysis.

For a long time it has been believed that the decay of MPO-I is a spontaneous process which can be described by first-order kinetics (7-9). Experimental results in this study indicate that this decay is not totally spontaneous; both hydrogen peroxide and superoxide are reactants. However, the kinetic analysis of the decay of MPO-I to MPO-II still suggests the presence of a spontaneous component, that is, an autodecay pathway for MPO-I. Impurities in the solution may contribute to the decay of MPO-I, but the extent must be very limited, as evidenced in a study in which the repeated addition of hydrogen peroxide had little effect on the decay of MPO-I (9). There have been speculations that MPO-I is such a strong oxidizing agent that one or more amino acid residues of the protein moiety of the enzyme may become suitable substrates (10) and donate one electron to the porphyrin π-cation

radical of MPO-I. Future experiments can be performed to test this hypothesis. For example, a pH profile of the decay of MPO-I to MPO-II would yield information of the possible amino acid residue(s) involved in the reduction of MPO-I.

The poor stability of compound I appears to be a common feature for almost all peroxidases, especially for animal peroxidases. Except for a few cases in which the reason has been found (e.g., the instability of chloroperoxidase can mainly be attributed to its well established catalase activity (11,12)), the mechanism of compound I decay for most peroxidase is still a riddle. Experiments on other peroxidases parallel to that on MPO carried out in this study would be interesting. From these experiments, a general picture of reactions of peroxidases with H_2O_2 could be obtained, which in turn would contribute to our understanding of the diversified bio-functions of peroxidase systems.

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APPENDIX I

COMPUTER SIMULATION OF CONCENTRATION TIME COURSES OF MPO, MPO-I AND MPO-II

The mechanism proposed in chapter six and seven can be rewritten here as following:

$$MPO + H_2O_2 \underset{k_{1b}}{\overset{k_{1f}}{\rightleftharpoons}} MPO\text{-}I$$

$$MPO\text{-}I + H_2O_2 \xrightarrow{k_2} MPO\text{-}II + O_2^{\cdot-}$$

$$MPO\text{-}I \xrightarrow{k_2^0} MPO\text{-}II$$

$$MPO\text{-}I + O_2^{\cdot-} \underset{k_{3b}}{\overset{k_{3f}}{\rightleftharpoons}} MPO\text{-}I\bullet\bullet\bullet O_2^{\cdot-}$$

$$MPO\text{-}I\bullet\bullet\bullet O_2^{\cdot-} \xrightarrow{k_4} MPO\text{-}II + O_2$$

The following rate equations are derived based on the above reactions:

$$\frac{d[MPO]}{dt} = -k_{1f}[MPO][H_2O_2] + k_{1b}[MPO\text{-}I]$$

$$\frac{d[H_2O_2]}{dt} = -k_{1f}[MPO][H_2O_2] + k_{1b}[MPO\text{-}I] - k_2[H_2O_2][MPO\text{-}I]$$

$$\frac{d[MPO\text{-}I]}{dt} = k_{1f}[MPO][H_2O_2] - k_{1b}[MPO\text{-}I] - k_2[H_2O_2][MPO\text{-}I] - k_2^0[MPO\text{-}I] - k_{3f}[MPO\text{-}I][O_2^{\cdot-}] + k_{3b}[MPO\text{-}I\bullet\bullet\bullet O_2^{\cdot-}]$$

$$\frac{d[\text{MPO-I}\bullet\bullet\bullet O_2^-]}{dt} = k_{3f}[\text{MPO-I}][O_2^-] - k_{3b}[\text{MPO-I}\bullet\bullet\bullet O_2^-] - k_4[\text{MPO-I}\bullet\bullet\bullet O_2^-]$$

$$\frac{d[\text{MPO-II}]}{dt} = k_2[H_2O_2][\text{MPO-I}] + k_2^0[\text{MPO-I}] + k_4[\text{MPO-I}\bullet\bullet\bullet O_2^-]$$

$$\frac{d[O_2^-]}{dt} = k_2[H_2O_2][\text{MPO-I}] - k_{3f}[\text{MPO-I}][O_2^-] + k_{3b}[\text{MPO-I}\bullet\bullet\bullet O_2^-]$$

Utilizing the mass balance for enzyme species

$$[\text{MPO}] + [\text{MPO-I}] + [\text{MPO-II}] + [\text{MPO-I}\bullet\bullet\bullet O_2^-] = [\text{MPO}]_0 = a$$

and introducing y_i (i = 1,2,3,4,5) to represent the relative concentrations

$$y_1 = \frac{[H_2O_2]}{a}$$

$$y_2 = \frac{[\text{MPO-I}]}{a}$$

$$y_3 = \frac{[\text{MPO-I}\bullet\bullet\bullet O_2^-]}{a}$$

$$y_4 = \frac{[\text{MPO-II}]}{a}$$

$$y_5 = \frac{[O_2^-]}{a}$$

We obtain

$$\dot{y}_1(t) = -a k_{1f} y_1 + k_{1b} y_2 + a(k_{1f} - k_2) y_1 y_2 + a k_{1f}(y_1 y_3 + y_1 y_4)$$

$$\dot{y}_2(t) = a^2 k_{1f} y_1 - (k_{1b} + k_2^0) y_2 - a(k_{1f} + k_2) y_1 y_2 - a k_{1f}(y_1 y_3 + y_1 y_4)$$

$$- a k_{3f} y_2 y_5 + k_{3b} y_3$$

$$\dot{y}_3(t) = a k_{3f} y_2 y_5 + (k_{3b} + k_4) y_3$$

$$\dot{y}_4(t) = a k_2 y_1 y_2 + k_2^0 y_2 + k_4 y_3$$

$$\dot{y}_5(t) = a k_2 y_1 y_2 - a k_{3f} y_2 y_5 + k_{3b} y_3$$

With the initial concentrations being

$y_1(0) = b$

and

$y_2(0) = y_3(0) = y_4(0) = y_5(0) = 0,$

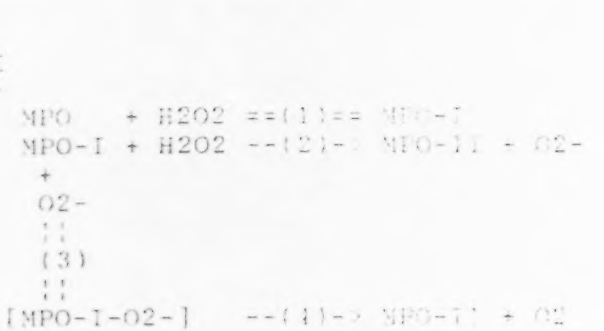
the time course of concentrations can be obtained by solving the differential equations digitally. The following BASIC program for this purpose was written based on the Runge-Kutta method:

```
100 REM   PROGRAM FOR DIGITAL SOLUTION OF TIME COURSES OF MPO/H2O2 SYSTEM
110 LPRINT "REACTION SCHEME"
120 LPRINT "---------------"
130 LPRINT " "," MPO   + H2O2 ==(1)== MPO-I"
140 LPRINT " "," MPO-I + H2O2 --(2)-> MPO-II + O2-"
150 LPRINT " "," +"
160 LPRINT " "," O2-"
170 LPRINT " "," !!"
180 LPRINT " "," (3)"
190 LPRINT " "," !!
200 LPRINT " "," [MPO-I-O2-]   --(4)-> MPO-II + O2"
210 LPRINT
220 LPRINT
230 LPRINT "PARAMETERS  INPUT"
240 LPRINT "-----------------"
250 READ N,H,M,k1f,k1b,k20,k2,k3f,k3b,k4,a,b
260 LPRINT "_____________________________________________________________________"
270 LPRINT
280 LPRINT "k1f","k1b","k20","k2","k3f"
290 LPRINT k1f,k1b,k20,k2,k3f
300 LPRINT "---------------------------------------------------------------------"
310 LPRINT "k3b","k4","[MPO](M)","[H2O2]/[MPO]"
320 LPRINT k3b,k4,a,b
330 LPRINT "_____ _____ _____ _____________________________________________________"
340 LPRINT
350 LPRINT
360 LPRINT "TIME COURSES (in ratios to the initial enzyme concentration)"
370 LPRINT "-----------------------------------------------------------"
380 DIM Y(N),YY(N),Y0(N),F(N),HH(5)
390 READ X
400 FOR I=1 TO N
        READ Y(I)
        NEXT
410 PRINT "t(ms)","[MPO-I]","[MPO-I-O2-]","[MPO-II]"," [O2-]"
420 PRINT
430 FOR I=1 TO M
        GOSUB 500
440 IF (I<>INT(I/250)*250) THEN 460
450 PRINT INT(X*1000+0.5),INT(Y(2)*100000+0.5)/100000,INT(Y(3)*100000+0.5)/10000
0,INT(Y(4)*100000+0.5)/100000,INT(Y(5)*100000+0.5)/100000
460 NEXT I
470 END
480 DATA 5,0.001,5000,2.8E7,20,.5,1.6E5,2.5E9,100,.1,4E-7,50
490 DATA 0,50,0,0,0,0
```

```
500 X0=X
510 FOR II=1 TO N
        Y0(II)=Y(II)
        YY(II)=Y(II)
        NEXT II
515 HH(1)=H/2
    HH(2)=HH(1)
    HH(3)=H
    HH(4)=HH(3)
    HH(5)=HH(1)
520 FOR JJ=1 TO 4
530 GOSUB 800
535 X=X0+HH(JJ)
540 FOR II=1 TO N
        YY(II)=YY(II)+F(II)*HH(JJ+1)/3
560 Y(II)=Y0(II)+F(II)*HH(JJ)
    NEXT II

570 NEXT JJ
580 FOR II=1 TO N
        Y(II)=YY(II)
        NEXT II

590 RETURN

800 F(1)=a*(-k1f*Y(1)+k1b*Y(2)/a+(k1f-k2)*Y(1)*Y(2)+k1f*(Y(1)*Y(3)+Y(1)*Y(4)))
810 F(2)=a*(k1f*Y(1)-(k1b+k20)*Y(2)/a-(k1f+k2)*Y(1)*Y(2)-k1f*(Y(1)*Y(3)+Y(1)*Y(
))-k3f*Y(2)*Y(5)+k3b*Y(3)/a)
820 F(3)=k3f*a*Y(2)*Y(5)-(k3b+k4)*Y(3)
830 F(4)=k20*Y(2)+k2*a*Y(1)*Y(2)+k4*Y(3)
840 F(5)=k2*a*Y(1)*Y(2)-k3f*a*Y(2)*Y(5)+k3b*Y(3)

850 RETURN
```

A typical computer printout is presented below which shows the time courses for the concentrations of compound I, compound I — superoxide complex, superoxide, and compound II, calculated based on the input of rate constants and initial concentrations:

REACTION SCHEME



PARAMETERS INPUT

k1f	k1b	k20	k2	k3f
2.8E+07	20	.5	160000	2.5E+09

k3b	k4	[MPO](M)	[H2O2]/[MPO]
100	.1	.0000004	20

TIME COURSES (in ratios to the initial enzyme concentration)

t(ms)	[MPO-I]	[MPO-I-O2-]	[MPO-II]	[O2-]
100	.71604	.08183	.13395	.01246
200	.55949	.14339	.2436	.02657
300	.44185	.18554	.33021	.04281
400	.35348	.21298	.39954	.06092
500	.287	.22944	.4559	.08044
600	.23677	.23792	.50247	.10081
700	.19848	.24077	.54159	.12146
800	.16895	.23975	.57499	.1419
900	.14583	.23617	.60391	.16179
1000	.12746	.23094	.62927	.1809
1100	.11263	.22469	.65178	.1991
1200	.10047	.21786	.67195	.21635
1300	.09036	.21073	.69016	.23264
1400	.08185	.2035	.70673	.248
1500	.07459	.19629	.7219	.26247
1600	.06834	.18919	.73586	.27609
1700	.0629	.18225	.74875	.28894
1800	.05814	.17551	.76072	.30105
1900	.05393	.16898	.77186	.31248
2000	.05018	.16267	.78228	.32328

2100	.04682	.15661	.78203	.33351
2200	.04381	.15076	.80118	.34318
2300	.04108	.14514	.8098	.35233
2400	.0386	.13974	.81792	.36101
2500	.03634	.13455	.82559	.36925
2600	.03427	.12957	.83284	.37708
2700	.03236	.12479	.83971	.38452
2800	.03061	.1202	.84622	.3916
2900	.02899	.11579	.8524	.39834
3000	.02749	.11156	.85828	.40477
3100	.02609	.1075	.86388	.41089
3200	.02479	.1036	.8692	.41674
3300	.02358	.09985	.87428	.42232
3400	.02245	.09625	.87912	.42765
3500	.02138	.09279	.88375	.43275
3600	.02039	.08947	.88816	.43763
3700	.01945	.08627	.89235	.44229
3800	.01857	.0832	.89642	.44676
3900	.01774	.08024	.90029	.45103
4000	.01696	.0774	.90399	.45513
4100	.01622	7.466999E-02	.90753	.45906
4200	.01552	.07204	.91093	.46282
4300	.01486	.06951	.91418	.46643
4400	.01424	.06707	.91731	.4699
4500	.01364	.06473	.9203	.47323
4600	.01308	.06247	.92318	.47642
4700	.01254	.0603	.92594	.47949
4800	.01203	.0582	.9286	.48244
4900	.01155	.05618	.93114	.48527
5000	.01109	.05424	.93359	.488

Normal termination. Press any key.

On the other hand, the real time courses of compounds I and II concentrations were obtained from the corresponding stopped flow traces through absorbance calibration. Therefore, by assuming and adjusting the values of rate constants for reaction between compound I and superoxide, the concentration time courses were calculated and adjusted to simulate the real concentration time courses. The values for those rate constants were then estimated thereby using orthogonal array optimization analysis (Taguchi, G. in *System of Experiment Design,* Vols. 1 & 2, UNIPUB/Kraus International Publications, White Plain, New York, 1987.).

APPENDIX II

DERIVATION OF THE EXPRESSION OF THE APPARENT SECOND ORDER RATE CONSTANT FOR MPO-II FORMATION

The apparent second order rate constant for MPO-II formation from MPO-I and O_2^- is defined as following:

$$\text{MPO-I} + O_2^- \xrightarrow{k_{app}} \text{MPO-II} + O_2$$

The corresponding reactions in Scheme I can be rewritten here:

$$\text{MPO-I} + O_2^- \underset{k_{3b}}{\overset{k_{3f}}{\rightleftharpoons}} \text{MPO-I}\cdots O_2^- \xrightarrow{k_4} \text{MPO-II} + O_2$$

Thus the rate of MPO-II formation

$$v = k_{app}\,[\text{MPO-I}]\,[O_2^-]$$

$$= k_4\,[\text{MPO-I}\cdots O_2^-]$$

From the equilibrium involving $\text{MPO-I}\cdots O_2^-$, MPO-I and O_2, we know

$$\frac{[\text{MPO-I}]\,[O_2^-]}{[\text{MPO-I}\cdots O_2^-]} = K = \frac{k_{3b}}{k_{3f}}$$

Thus

$$[\text{MPO-I}\cdots O_2^-] = \frac{k_{3f}}{k_{3b}}\,[\text{MPO-I}]\,[O_2^-]$$

By replacing $[\text{MPO-I}\cdots\text{O}_2^-]$ with $[\text{MPO-I}]$ and $[\text{O}_2^-]$ in the rate equation for MPO-II formation, we obtain

$$v = k_{app}\,[\text{MPO-I}]\,[\text{O}_2^-]$$

$$= k_4 \frac{k_{3f}}{k_{3b}}\,[\text{MPO-I}]\,[\text{O}_2^-]$$

Therefore

$$k_{app} = \frac{k_4\,k_{3f}}{k_{3b}}$$